THE JOHN CURTIN SCHOOL OF MEDICAL RESEARCH

Division of Immunology and Genetics

On the Role of Cell-Mediated Cytotoxicity in a Mouse Model of Flavivirus Encephalitis

Rosa María Licón Luna

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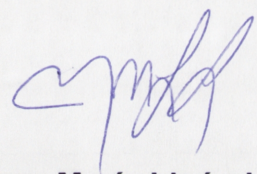
Statement

The author performed the work presented in this dissertation. The material herein has not been submitted, in whole or in part for a degree at this or any other University.



May 2004 **Rosa María Licón Luna**

Dedication

To my loving husband Hans, for his great support; with all my admiration for his special talent to find solutions, and for his positive attitude in general. To my children Igor and Vera, who are a great joy, and to Lena who will be born in a couple of weeks. With eternal gratitude to my parents Rodrigo and Manuela, and to Mutti Gertrud. To tío Isidro, whom I greatly admire and respect, and who has been a role model and a motivation for new challenges. The completion of this thesis is doubly satisfying, because I could combine writing with spending time with my parents in Mexico, and establishing my own family in Germany.

Table of Contents

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Abbreviations

2-ME	2-Mercaptoethanol
Ab	antibody
ADCC	antibody-dependent cytotoxicity
ADE	antibody-dependent enhancement
AE	Australian encephalitis
Ag	antigen
APC	antigen presenting cell
ATD	average time to death
BCR	B-cell receptor
BSA	bovine serum albumin
bp	base pair
β_2-M	β_2-microglobulin
C6/36	*Aedes albopictus* mosquito salivary gland cell line
cDNA	complementary DNA
CF	complement fixation
CMI	cell-mediated immunity
CNS	central nervous system
CPE	cytopathic effect
C protein	capsid protein
Cr	chromium
CSF	cerebrospinal fluid
DEN	dengue virus
DHF	dengue hemorrhagic fever
DTH	delayed-type hypersensitivity
DMEM	Dulbecco's minimum essential medium
DNA	deoxyribunocleic acid
dGTP	2'deoxiguanosine 5' triphosphate
DSS	dengue shock syndrome
E	envelope protein
EDTA	ethylenediemine tetra-acetic acid
ELISA	enzyme linked immunosorbent assay
EMEM	Eagle's minimum essential media with Earle's salt
E protein	envelope protein
E:T	effector to target ratio
ER	endoplasmic reticulum
FACS	fluorescence-activated cell sorter
Fas	Fibroblast associated
FasL	Fas ligand
FCS	fetal calf serum
FITC	fluorescein-isothiocyanate conjugated
gld	generalized lymphoproliferative disease
gzm	granzyme (granule enzyme)
h	hour
HBSS	Hanks' balanced salt solution
HEPES	N-2-Hydroxyethylpiperazine-N-2-ethanesulfonic acid
HI	heat-inactivated
ic	intracranial
IFN	interferon

Ig	immunoglobulin
IL	interleukin
ip	intraperitoneal
iv	intravenous
JEV	Japanese encephalitis virus
kD	kilodalton
KOK	Kokobera
KUN	Kunjin
LCMV	lymphocytic choriomeningitis virus
LGT	Langat
LMI	leukocyte migration inhibition
lpr	lymphoproliferative disease
mb	mouse brain MVE stock
MHC	major histocompatibility complex
MHV	murine hepatitis virus
min	minute
MML	Montana myotis leukoencephalitis virus
MOI	multiplicity of infection
M protein	membrane protein
MVE	Murray Valley encephalitis
N	number
ng	nanogram
NK	natural killer
nm	nanometer
NO	nitric oxide
NP	nucleoprotein
NS protein	nonstructural protein
nt	nucleotide
OD	optical density
ORF	open reading frame
PA	plaque assay
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
perf	perforin
PFU	plaque-forming units
pi	post-infection
pM	picamole
PNS	peripheral nervous system
POW	Powassian
prM protein	precursor to membrane
PSN	penicillin, streptomycin, neomycin
pvi	perivascular infiltration
RAG	recombination activating gene
RF	reading frame
RNA	ribonucleic acid
rt	room temperature
RT	reverse transcription
SCID	severe combined immune deficiency mutation
SEM	standard error of the mean
SF	Semliki Forest virus
STR	structural protein

SV	sindbis virus
rVV	recombinant vaccinia virus
SD	standard deviation
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SLE	Saint Louis encephalitis
SPF	specific pathogen free
TBE	thick-borne encephalitis
Tc	cytotoxic T cell
tc	tissue culture
T cells	thymus-derived lymphocytes
TCR	T-cell receptor
Th	T-helper
TK	thymidine kinase
TNF	tumor necrosis factor
U	unit
UTR	untranslated region
VD	virus diluent
V(D)J	Variable (Diversity) Joining
Vero	African green monkey kidney cell line
VSV	vesicular stomatitis virus
VV	vaccinia virus
WNV	West Nile virus
w	weeks
wt	wild-type
w/v	weight per volume
YF	yellow fever

Abstract

This thesis establishes important aspects of the pathogenesis of Murray Valley encephalitis virus (MVE) in C57Bl/6 mice as a basis to study the role of natural killer (NK) cell-mediated and cytotoxic T (Tc) cell-mediated cytotoxicity in encephalitic flavivirus infection, using mice of the same genetic background but with defined deficiencies in immune pathways. Virus was administered to six-week-old mice by the intravenous route, analogous to the hematogenous portal entry in natural infections, using a virus dose in the range experienced following the bite of an infectious mosquito. In this model, infection with 0.1 to 10^5 PFU of virus gave mortality in ~50% of the mice, despite low or undetectable virus growth in extraneural tissues (based on results from two assays, plaque titration in Vero cells and RT/PCR). In contrast to the absence of a dose-response in infections with 0.1-10^5 PFU of MVE, infection with 10^8 PFU resulted in direct virus entry into the brain and 100% mortality.

B- and T-cell deficient (RAG-1-/-) mice that were infected with MVE showed increased susceptibility, but delayed mortality, compared to wild-type mice. The increased susceptibility was most likely due to the lack of antibody, given that passive transfer of MVE-primed B cells to MVE-infected wild-type mice resulted in complete protection from the encephalitic disease. The delayed mortality of MVE-infected RAG-1-/- mice suggested that T cells contribute to pathology in MVE infection. This was corroborated by experiments in which $CD8^+$ T-cell deficient ($ß_2$-M-/-) mice were infected with MVE, which showed that these mice had a reduced and delayed mortality relative to infected wild-type mice. The deleterious role of $CD8^+$ T cells in this mouse model for flaviviral encephalitis was also apparent when mice with a deficiency in the cytolytic effector functions of Tc and NK cells were tested. When infected with MVE, the mortality of mice deficient in either the granule exocytosis- or Fas-mediated pathways of cytotoxicity was marginally delayed and reduced, relative to wild-type mice. To uncover a possible redundancy of the two cytolytic effector mechanisms in Tc or NK cell-mediated exacerbation of encephalitic disease, I used mice deficient in both granule exocytosis- and Fas-mediated cytotoxicity. These double-deficient mice were resistant to a low-dose peripheral infection with MVE, possibly as a result of reduced pathology at the blood–brain barrier in the absence of the cytotoxic functions of NK and Tc cells, which may have limited the spread of the neurotropic virus into the brain.

In summary, this investigation uncovered that NK and Tc cells do not make a beneficial contribution to the recovery from MVE infection, and highlights that, on the contrary, cell-mediated cytotoxicity can exacerbate disease in encephalitic viral infection.

Chapter 1

Background: flavivirus pathogenesis and the immune response

1.1 Introduction

Viruses have probably been around for as long as there have been cellular hosts for viruses to replicate in. Records of rabid dogs have been found in Mesopotamia, and in Egypt archaeologists found ancient drawings of people with polio. Smallpox played a key role in facilitating the European invasion of South and Central America, when the indigenous, immunologically-naïve, human population succumbed rapidly to the virus brought by the Europeans. Western Africa has long been regarded as the home of the yellow fever virus, although the first records of yellow fever date from an outbreak that occurred between 1483 and 1484, on the Yucatán Peninsula (Gongora-Bianchi, 2000). The sacred Mayan books relate how an epidemic of vomiting blood or *xekik* (Mayan name for yellow fever) occurred among the Indians, due to their proximity to monkeys. Interestingly, and with an anticipation of how more than 500 years later *Aedes aegypti* would be identified as the vector of the virus, the Mayan scripts relate how *xekik* is transmitted via mosquitoes. *Haemagogus* mosquitoes were responsible for yellow fever transmission in the pre-Columbian era. When the Spanish brought *A. aegypti* to the new world, new outbreaks of yellow fever were facilitated, giving the surviving conquistadores the color of the gold they went to search for (Gongora-Bianchi, 2000).

Early in the twentieth century, it was found that yellow fever (YF) was produced by a filterable agent that could be transmitted to humans by bites of the mosquito *A. aegypti* (Strode, 1951; Rice, 1996). Yellow fever was the first virus to be isolated in 1927 and the first virus to be cultivated *in vitro* in 1932 (Monath, 1996). The discovery of the YF virus (YF) led to the production of an effective and safe live vaccine (YF-17D). This vaccine has now been used for more than 60 years (Guirakhoo et al., 1999). It gives demonstrable lifelong immunity against YF occurs to over 95% of vaccinees, within 10 days (Monath, 1996).

The twentieth century, which opened with the promise of eradication of most infectious diseases, has closed with the specter of the emergence and re-emergence of many deadly infectious diseases (Berns and Rager, 2000). Some flaviviral diseases, along with many other arthropod-borne infections, have been spreading geographically in conjunction with ecological changes that have favored increased vector densities, such as dam construction, irrigation, urbanization, deforestation and increased global warming. Increased human travel has also contributed significantly to the spread of infectious agents, introducing them into areas in which they had hitherto been absent (Gubler, 1998; Gratz, 1999).

Flaviviruses are medically the most important of the arthropod-borne viruses, causing human and animal disease with worldwide distributions (Rice, 1996). The amplification cycle of arthropod-borne flaviviruses that cause encephalitis involves mosquitoes and ticks as vectors, and viremic vertebrate species as reservoirs. Infected humans are dead-end hosts, which do not participate in perpetuation of virus transmission. Vector species vary, but culicine mosquitoes (principally *Culex* spp.) and ticks (*Ixodes* spp.) are responsible for amplification cycles (Monath, 1996).

Yellow fever virus is the prototype of the family Flaviviridae (*flavus*, Latin for yellow). The family Flaviviridae comprises three genera, the flaviviruses, the hepatitis C viruses (from Greek *hepar*, *hepatos*, or liver), and the pestiviruses (from Latin *pestis,* or plague). These three genera have diverse biological properties and show no serological cross-reactivity, but are similar in terms of virion morphology, genome organization, and RNA replication strategy (Monath, 1996).

Among the genus flavivirus, 28 of the more than 70 known members have been associated with disease in humans. An evolutionary tree of flaviviruses, drawn on the basis of their envelope (E) protein amino acid sequence homologies, is presented in

Fig. 1.1. Infected hematophagous arthropod vectors (ticks or mosquitoes) transmit all flaviviruses that affect humans, which makes eradication of these viruses unlikely (Monath, 1996).

In humans, there are, broadly speaking, three clinical syndromes that are associated with the main pathogenic flaviviruses (e.g. YF, dengue (DEN), and Japanese encephalitis (JE)): 1) hemorrhagic fever; 2) fever with rash, often associated with myalgia and arthralgia; and 3) encephalitis and meningoencephalitis (Monath, 1996). The clinical spectrum of YF symptoms varies from a very mild, non-specific, febrile illness to a fulminating disease with pathognomonic features of jaundice and bleeding diathesis, with hepatorenal involvement. Infection with DEN produces a spectrum of clinical illness that ranges from a non-specific febrile syndrome to a severe and fatal hemorrhagic disease (Monath, 1996). Dengue is classified as an emerging disease, because its occurrence has increased in humans over the last 20 years (Tomori, 1999; Jelinek, 2000; Solomon and Mallewa, 2001; Werner, 2001). Japanese encephalitis virus (JEV) produces acute encephalomyelitis, and has a high fatality rate throughout Asia (Monath et al., 1999). West Nile virus (WNV) is the most widely distributed neurotropic flavivirus and a more 'benign' member of the JEV serogroup (Solomon and Mallewa, 2001).

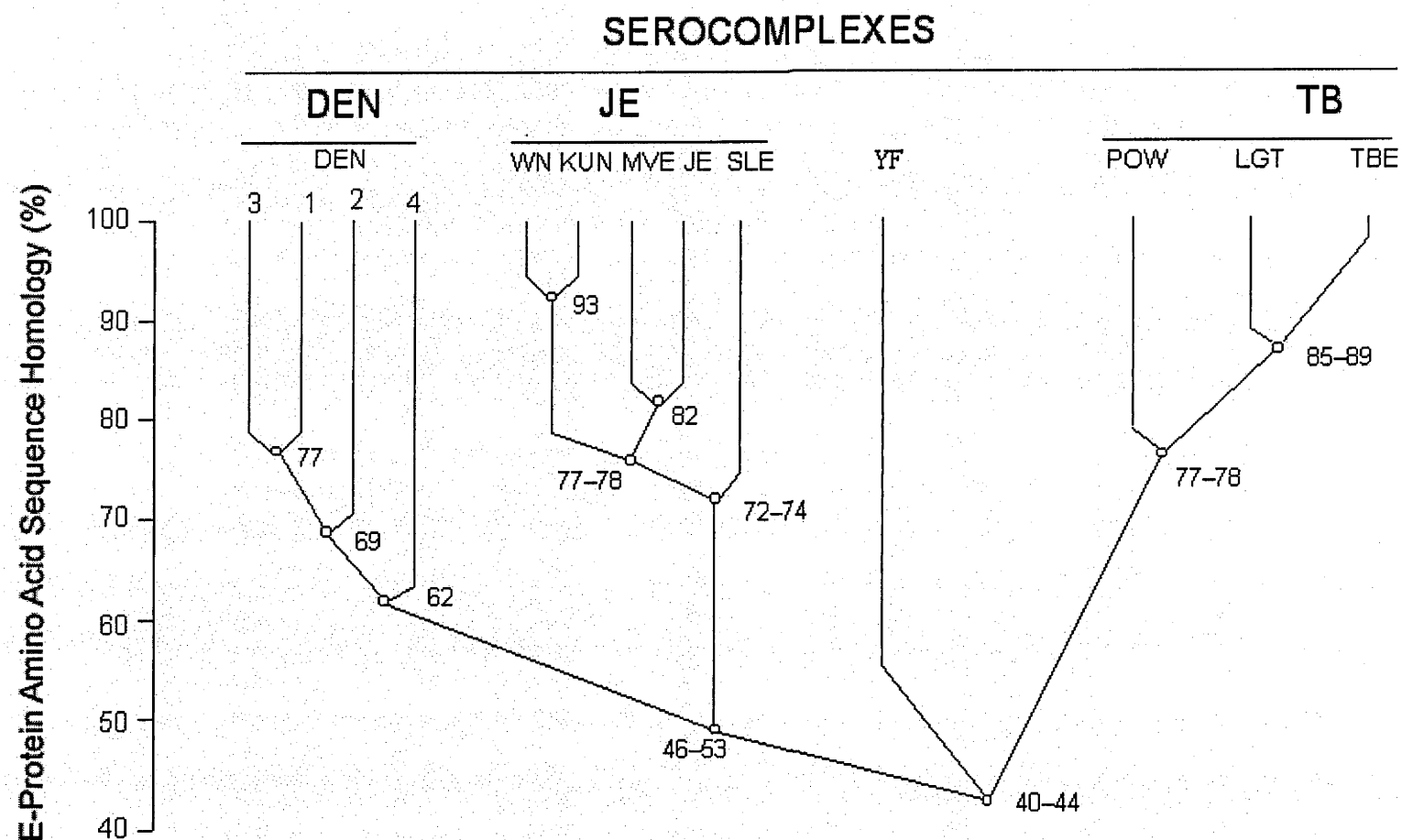

Fig. 1.1 Evolutionary tree of flaviviruses based on their envelope (E) protein homologies. DEN, dengue virus; JEV, Japanese encephalitis virus; WNV, West Nile virus; KUN, Kunjin virus; MVE, Murray Valley encephalitis virus; SLE, Saint Louis encephalitis virus; YF, yellow fever virus; POW, Powassian virus; LGT, Langat virus; TBE, tick-borne encephalitis virus. Modified from (Monath, 1996).

WNV infects humans, horses and birds in Africa, the Middle East, and southwestern Asia, and has recently (1999) been identified for the first time in North America (New York) (Briese et al., 1999; Jia et al., 1999). The outbreak of WNV in New York involved 50 persons who showed signs such as fever, arthralgia and rash. There were 5 fatal cases of acute encephalitis in elderly patients. This virus is now enzootic in the United States (Ostlund et al., 2000). It is likely that WNV will keep spreading across the American continent as it follows the migratory patterns of the reservoir birds (Petersen et al., 2002). A similar spread occurred in Canada, Mexico and in the Caribbean Islands (Diamond et al., 2003a).

Aside from the successful vaccine against the prototype flavivirus YF, vaccines against two other flaviviruses, JEV and tick-borne encephalitis (TBE) virus, are also available today (Cardosa, 1998). There are no approved vaccines against the rest of the flaviviruses. To produce effective vaccines against other flaviviruses (including DEN), much more effort has to be made to understand the processes of pathogenesis and the role of antiflaviviral immunity in protection and recovery from infection. The therapeutic treatment is primarily supportive, since there are no specific measures of proven efficacy (Cardosa, 1998).

1.1.1 Flaviviruses of major concern worldwide

Yellow fever

Despite landmark achievements in the understanding of the epidemiology of YF, and despite the availability of a safe and efficacious vaccine, the YF virus is still an important cause of hemorrhagic fever and related mortality, mainly among populations in the Americas and Africa. It affects an estimated 200,000 persons and causes close to 30,000 deaths per year, and it poses a threat to unvaccinated populations (Monath, 1987; Leyssen et al., 2000). Yellow fever is a zoonotic disease that has three transmission cycles with various combinations of transmission between monkeys, mosquitoes, and humans. It produces an acute disease, which is characterized by a sudden onset, and has two phases of development that are separated by a short period of remission. The primary transmission cycle involves wild non-human primates and various sylvatic (tree-hole-breeding) aedine mosquitoes. The second transmission cycle involves humans, who are tangentially exposed when they encroach on the sylvatic cycle. Finally, the urban transmission cycle occurs when YF is spread from human to human through the mosquito vectors (Monath, 1996; Chen and Cosgriff, 2000).

Dengue

The geographical distribution of DEN has increased, and it has become numerically the most important arboviral disease in humans. This global problem affects approximately 100 million people per year (Solomon and Mallewa, 2001). The first reported epidemics of dengue fever occurred between 1779 and 1780 in Asia, Africa, and North America. The near-simultaneous occurrence of outbreaks on these three continents indicates that these viruses and their mosquito vectors (mainly *A.*

aegypti) have had a worldwide distribution in the tropics for more than 200 years. Dengue was the second human disease, after YF, to be attributed to a 'filtrable virus' (Solomon and Mallewa, 2001).

The origin of the word dengue is currently attributed to the Swahili phrase *ka dinga pepo* where *ka* means 'a kind of...', *dinga* means 'sudden cramp-like seizure' and *pepo* means 'plague'. In Cuba, this phrase was popularly replaced by the hispanicized word dengue (Rigau-Perez, 1998).

Classical dengue fever (DEN), dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS) are all caused by one or more of the four closely related, but antigenically distinct, virus serotypes (DEN 1–4). Infection by one of these serotypes does not provide long-lasting cross-protective immunity against the others (Rothman and Ennis, 1999). However, new research shows that a vaccine against all four types of DEN appears to induce immune responses for all four viruses (Rothman et al., 2001). An effective dengue vaccine must induce antibodies against all the viruses, because a person who has developed antibodies against one of the serotypes is at risk of developing dengue hemorrhagic fever if infected with any of the other three serotypes (Kanesa-thasan et al., 2001; Rothman et al., 2001; Sabchareon et al., 2002). DEN viruses generally cause a benign syndrome with self-limited febrile illness, although more acute signs can occur when multiple infections with serotypes 1 through 4 lead to antibody-dependent enhancement (ADE) of infection. The spread of the four DEN virus serotypes has lead to the increased incidence of DHF, with approximately 2.5 billion people at risk. DHF is an acute, potentially life-threatening, capillary leak syndrome and is more common in secondary than in primary dengue virus infections. Important risk factors for DHF include the strain and serotype of the infecting virus, as well as the age, immune status, and genetic predisposition of the patient (Rothman and Ennis, 1999).

Tropical and subtropical areas of many countries are now hyperendemic (i.e. they have a prevalence of two or more serotypes), which enhances the occurrence of DHF and DSS (Gubler, 1998).

Japanese encephalitis

JEV is the leading cause of lethal arthropod-borne human encephalitis worldwide (Ogata et al., 1991; Monath, 1996), with 50,000 cases and 10–15,000 deaths per year (Solomon and Mallewa, 2001). In Japan, a disease that resembles JE was recognized in horses and humans as early as 1871, and, in 1924, a filterable agent was extracted from human brain during a fulminating epidemic. It was not until 1935 that scientists virologically and serologically established the virus as being the prototype (Nakayama) strain of JEV, which is also the prototype of the encephalitic group of flaviviruses (Table 1.1). The virus was isolated in 1938 from Culex tritaeniorhynchus, and birds and pigs were recognized as the principal viremic hosts (Monath, 1996).

In Asian countries, rice-field-breeding culicine mosquitoes are the principal vectors of JEV. Children are primarily affected in endemic areas, whereas older age groups may not show any infection, or have infections that may be less severe because they have life-long protective immunity due to childhood exposure to the virus (Monath, 1996).

Table 1.1 The Japanese encephalitis – West Nile complex of flaviviruses[a]

Virus	Acronyms	First isolated	Geographical range	Vectors	Reservoirs
St. Louis encephalitis	SLE	1933	North, Central, and South America	*Culex pipiens* *C. p. quinquefasciatus*	Birds
West Nile	WNV	1937	southern Europe, Africa, Mediterranean, western and southern Asia, North America	*Culex univittatus* *C. antenatus* *C. molestus* *C. vishnui*	Birds
Japanese encephalitis	JEV	1935	eastern India, east and South-East Asia, PNG, northern Australia	*Culex tritaeniorhynchus* *C. gellidus* *C. vishnui*	Birds
Murray Valley encephalitis	MVE	1951	Australia, New Guinea, west Iran	*Culex annulirostris*	Birds

[a] Summarized from (Monath, 1996).

There is no specific treatment for JE or any other flaviviral disease (Johnson et al., 1985), however, some patients react well to ventilation support since respiratory paralysis could occur during encephalitic flavivirus infections (Tzeng, 1989). Vector control and vaccination are still the most effective control measures. The mouse brain vaccine, produced by the Research Foundation for Microbiological Diseases (Biken) in Osaka, Japan, has been in wide use since the 1960s. The presence of the JEV in Japan has not been diminished, as is shown by the data of antibody acquisition among pigs in Japan, but JE vaccination has probably contributed to the low incidence of infections among children. Cases of JE have also occurred among travelers to Asia; thus, vaccination is recommended before traveling (Sturchler and Steffen, 2001; Monath et al., 2002; Kirkpatrick and Alston, 2003).

1.1.2 Flaviviruses in Australia

Flaviviruses endemic to Australia

The main Australian flaviviruses are Murray Valley encephalitis (MVE), Kunjin (KUN) and Kokobera (KOK) are described below. Three other Australian flaviviruses, of lesser public health impact, have also been related to human infection: Alfuy and Stratford, two additional members of the JEV serocomplex, and Edge Hill, a member of the Uganda serological group (Mackenzie et al., 1994; Monath, 1996).

Murray Valley encephalitis

MVE is the major etiological agent of Australian encephalitis. Its similarity of symptoms in the clinical hosts to those of JEV, and its nucleotide sequence homology of 71% make it closely related to JEV (Kimura-Kuroda and Yasui, 1986; McAda et al.,

1987; Lobigs et al., 1988; Lee et al., 1990). In 1917, the disease was first described as Australian X disease, after an epidemic of encephalitis that occurred in eastern Australia in that year (Monath, 1996). Another major epidemic occurred in 1950–1951, in the Murray Valley area of southeastern Australia, during which the virus was first isolated from the brains of three fatal human cases (French, 1952). The first MVE mosquito isolates were obtained from *Culex annulirostris* in 1960 (Mackenzie et al., 1994). The predominant vertebrate hosts are thought to be migrating water birds, although certain domestic and wild mammals (including rabbits and kangaroos) have also been implicated on serological grounds (Mackenzie et al., 1994). The last major epidemic of MVE occurred in 1974, with 58 reported cases, 13 of which were fatal (Marshall, 1988). Currently, only sporadic cases occur in the Murray Valley area, however, there is a regular incidence of MVE in northern Australia, where cases of encephalitis often coincide with the increased mosquito activity during the December–June period (reviewed in (Marshall, 1988)). The most serious cases occur among aboriginal children, which may indicate that if the disease occurs early in life, it is more likely to be severe. People (of all ages) who have recently entered this region also seem to be susceptible to MVE infection (Mackenzie et al., 1993). While only one in about 1000 infected individuals develops encephalitis, clinical cases have an approximate 25% mortality, and 50% of survivors have significant neurological sequelae (Broom et al., 2000). The severity of the illness can range from fever to coma and death. Sequelae include paraplegia, impaired gait and motor coordination, and intellectual dysfunction (Monath, 1996).

Kunjin

KUN was first isolated from *Culex annulirostris* mosquitoes in 1960. KUN is less common than MVE, and produces a milder disease pattern in humans and horses (Hall et al., 2001). The clinical disease resembles MVE, although it has also been associated with non-encephalitic symptoms, involving fever and malaise (sometimes with joint involvement). The non-encephalitic symptoms occur more frequently than the traditional encephalitic symptoms (Mackenzie et al., 1994). The first case of KUN in humans was reported during a small outbreak of encephalitis in Western Australia in 1978, although KUN had already been implicated retrospectively as the causative agent of encephalitis in five cases from the last major MVE epidemic in 1974 (Doherty et al., 1976). Although KUN shares a number of biological and epidemiological characteristics with MVE (Marshall, 1988), its genomic sequence has a very high level of homology to that of WNV, which implies that it has a close evolutionary relationship with WNV (Coia et al., 1988). Moreover, recent phylogenic analyses show that some strains of WNV (including isolates from New York, Malaysia and Africa) are more similar to KUN virus and form a separate lineage to other WNV strains (Hall et al., 2001). These findings are in agreement with the Seventh Report of the International Committee for the Taxonomy of Viruses that designates KUN as a subtype of West Nile (Hall et al., 2001).

Kokobera

KOK is the third most important of the Australian flaviviruses in terms of human health (Mackenzie et al., 1994). The virus was first isolated from *Culex annulirostris* mosquitoes in northern Queensland in 1960 (Monath, 1996). Its vertebrate

hosts are believed to be macropods (wallabies and kangaroos), although horses may also play a minor role as hosts (Mackenzie et al., 1994). The first report in humans that indicated that KOK had pathogenic potential was compiled from three patients from southeastern Australia, who suffered acute polyarticular illnesses in the summer months of 1983–1985. Kokobera virus was isolated in the dry season; an interesting finding, which suggests that the virus is capable of year-round survival in vector-vertebrate cycles (Mackenzie et al., 1994).

Flaviviruses introduced into Australia

Dengue

Although epidemics of dengue fever were common in Australia in the late 19th century, and DHF was first described in Australia in 1898 (Blok et al., 1988), the evidence that is presently available suggests that dengue is not endemic in Australia and that epidemics arise from virus introduced by viremic travelers (Mackenzie et al., 1994; Mackenzie et al., 1998b). Dengue disappeared from Australia after an epidemic that occurred between 1953 and 1955 and reappeared in 1981 as a dengue type 1 epidemic, which affected 196 individuals. The most common symptoms were fever, headache, rash, myalgia, skin itching and arthralgia. Hemorrhagic manifestations were noted in 14 cases, but most other cases only showed skin petechiae. One individual also had bleeding from the gastrointestinal and urinary tracts. No cases of shock were recorded (Guard et al., 1984).

In 1993, an epidemic caused by dengue virus type 2 occurred in North Queensland (McBride, 1988). An analysis of symptoms was undertaken using a random

sample of 1000 residents, to determine the symptoms and the subclinical infection rate, and to establish the true extent of the epidemic. Diagnoses of dengue fever were based on the presence of both serum dengue 2 – neutralizing antibody and the presence of symptoms consistent with DEN infection. Approximately 20% of the population had been exposed to dengue and the rate of subclinical infections was 15%. Bleeding occurred more frequently in people who recalled that they also had a dengue infection 12 years earlier during the dengue 1 epidemic (McBride, 1988). If Australia follows the pattern that has been observed elsewhere in the world, cases of DHF may again be recorded on Australian soil in the foreseeable future (McBride, 1999).

Japanese encephalitis

JEV was first identified in Australia in 1995, with the diagnosis of three clinical cases (two fatal) among residents on Badu Island in the Torres Strait, northern Queensland (Hanna et al., 1996; Hall et al., 2001). JEV activity was more widespread in north Queensland in the 1998 wet season than in the three previous wet seasons, but in that year the ecological circumstances (e.g. less intensive pig husbandry, fewer mosquitoes) appear to have limited the transmission on the mainland (Hanna et al., 1999). Shortly after JEV activity was detected in humans and sentinel pigs on Badu Island in 1998, adult mosquitoes were collected, and 43 isolates of JEV were recovered. Researchers observed that there was between 99.1% and 100% nucleotide sequence identity between the 1995 and 1998 isolates of JEV from Badu Island. They also found that isolates of JEV from mosquitoes collected in Papua New Guinea in 1997 and 1998 showed similar percentages of identity to the 1995 isolates. Circumstantial evidence therefore suggests that cyclonic winds carried the infected mosquitoes from Papua New

Guinea to the Torres Strait islands and the Australian mainland (Hanna et al., 1999; Johansen et al., 2001).

1.2 Molecular characterization of flaviviruses

Flaviviruses are small and spherical (40–60 nm in diameter), and are surrounded by a host-derived lipid envelope, which encloses a single-stranded RNA of positive polarity (Westaway et al., 1985; Chambers et al., 1990; Rice, 1996) (Fig. 1.2). The viral envelope has a fringe of spike projections of about 5 nm (Rice, 1996). The most notable feature of the flavivirus genome is the presence of a single open reading frame (ORF), 10–11 kb long, which codes for 3380–3433 amino acids (Chambers et al., 1990; Rice, 1996; Heinz and Allison, 2000) and is flanked by noncoding regions at its 5' and 3' ends (Fig. 1.3). The RNA genome, type-1 capped at the 5' terminus, comprises untranslated regions (UTR), which contain conserved elements that are potentially involved in secondary structure formation (Rice, 1996). The nucleotide sequence of the 5' UTR is partially conserved in members of specific antigenic serocomplexes — the 3' UTR lacks a poly (A) tract, terminates with the conserved dinucleotide CU, and contains several conserved sequences that are specific for arthropod-borne viruses (Rice et al., 1986; Rice, 1996). For the mosquito-borne flaviviruses, the conserved RNA sequences CS1 and CS2 are located 5' to the 3' terminal secondary (stem and loop) structure. CS1 is approximately 26 nucleotides (nt) long and is partially complementary to a sequence near the 5' end in the region that encodes the capsid protein, suggesting that the genomic RNA can circularize. CS2 is approximately 24 nt long and is located 12 to 22 bases 5' upstream of CS1 (Rice et al., 1986; Rice, 1996). The genomic RNA is the only viral RNA found in infected cells, and its translation gives rise to a polyprotein which is

cotranslationally and posttranslationally processed into three viral structural proteins and seven non-structural proteins (Fig. 1.3) (Heinz and Allison, 2000).

The structural genes, which encode components of the virion, namely the capsid (C) protein, the membrane protein (prM/M) and the envelope glycoprotein (E), occupy one fourth of the ORF at its 5' end. The sequence homology of C is low, although the presence of (+) charged residues in C is conserved in the different flaviviruses. The glycosylated precursor of the structural M protein (prM) undergoes a delayed cleavage to M and is found on intra-cellular virions, indicating that cleavage (furin-mediated) occurs immediately before, or together with, virion release. The envelope (E) protein is the largest structural protein. It consists of approximately 500 amino acids in three antigenic domains (Heinz and Allison, 2000; Solomon and Mallewa, 2001) and has a molecular weight of 53 to 54 kD. As in other enveloped viruses, the E protein in flaviviruses is thought to be principally involved in virion attachment (by binding to cell surface receptors) and in fusion, resulting in the release of the nucleocapsid into the cytoplasm (Mathews et al., 1992).

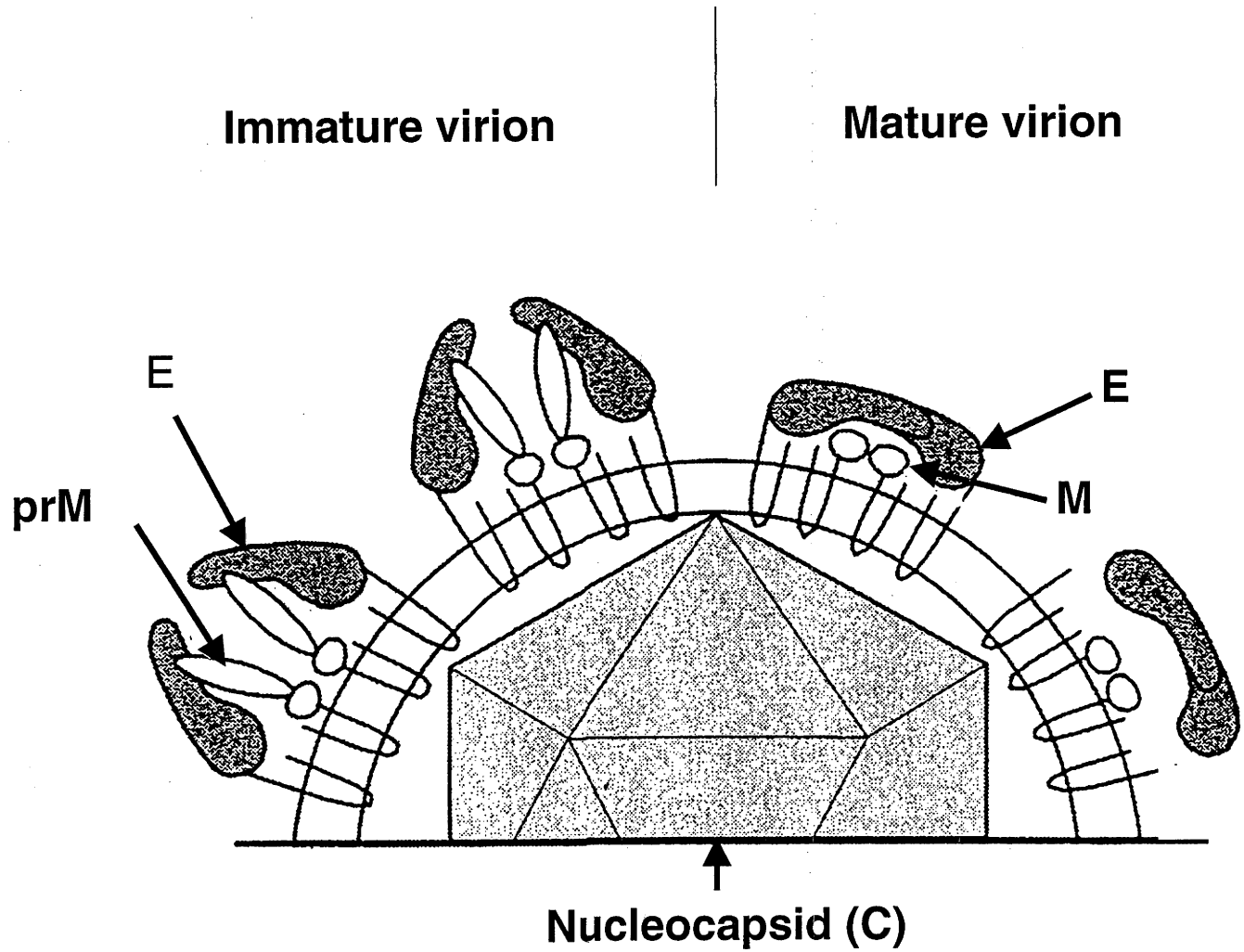

Fig. 1.2 Schematic representation of immature and mature flaviviruses. To determine their structure, limited trypsin digestion of TBE virus yielded a soluble dimeric ectodomain fragment of the E protein. Adapted from (Heinz and Allison, 2000).

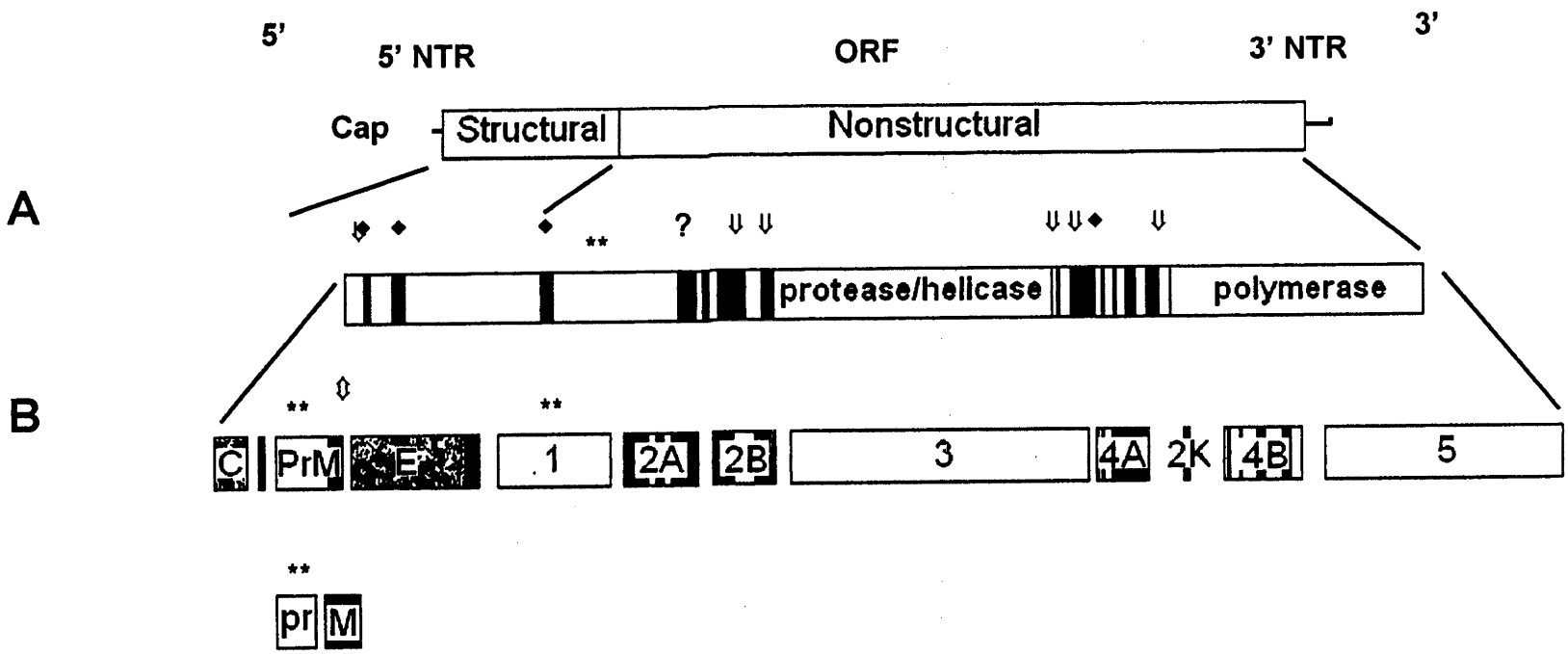

Fig. 1.3 Translation and processing of the flavivirus protein. **A.** Viral genome with the structural and nonstructural protein coding regions, the 5' cap, putative 3' secondary structure, and the 5' and 3' NTRs as labeled. **B.** Boxes indicate precursors and mature proteins that were generated by the proteolytic processing cascade. Mature structural proteins are indicated by shaded boxes, and the nonstructural proteins and structural protein precursors by open boxes. Contiguous stretches of uncharged amino acids are shown in black bars. Asterisks denote proteins with N-linked glycans but do not necessarily indicate the position or the number of sites used. Cleavage for host signalase (◆), the viral serine protease (⇓), furin or other Golgi-localized protease (⇳), or unknown proteases (?) are indicated. Adapted from (Rice, 1996).

Most of the ORF (75%) encodes the non-structural (NS) proteins. The majority of these proteins are required for viral replication: NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5. The non-structural 1 (NS1) glycoprotein is membrane-associated and plays an essential role in RNA replication (Lindenbach and Rice, 1997). NS2A has a role in RNA replication. It colocalizes with double-stranded RNA in cytoplasmic foci and binds strongly to the 3' UTR of the RNA, NS3 and NS5 (Rice, 1996; Mackenzie et al., 1998a). NS2A has been implicated in the JEV-induced cytopathic effect (CPE) (Chang et al., 1999). The work of Kümmerer and Rice (Kummerer and Rice, 2002) has shown that NS2A and NS3 play a role in the assembly and/or release of infectious YF particles. The NS2B protein is essential for viral replication; it is a component of the NS2B-3 protease and is thought to function in polyprotein cleavage, in association with the NS3 protein. NS3 is the second-largest viral protein (Rice, 1996; Scaramozzino et al., 2002). Sequence comparisons and biochemical analyses suggest that NS3 may be trifunctional, and may have protease, helicase and RNA triphosphatase activities (Rice, 1996). The NS3 protease activity is required for C protein cleavage, which triggers signalase cleavage of prM and secretion of spike heterodimer (Lobigs, 1993; Rice, 1996; Droll et al., 2000; Scaramozzino et al., 2002). NS4A binds strongly to most of the other non-structural proteins. NS4A binds to NS1 as a determinant of replicase function (Lindenbach and Rice, 1999). NS2B and NS4B (a transmembrane protein) are poorly conserved membrane-associated proteins whose functions are currently unclear, although it has been suggested that they may serve to anchor the viral replicase to cellular membranes (Chu and Westaway, 1992; Lindenbach and Rice, 1999). The strong association of the viral polymerase complex with the cell membrane suggests that NS2B, NS2A and NS4B may be necessary for localization and activity of the polymerase complex (Rice et al., 1985; Rice et al., 1986). NS5 is the largest and most

highly conserved flavivirus protein. NS5 is thought to function as the viral RNA polymerase, and possesses a GDD motif, which it has in common with RNA-dependent RNA polymerases of other positive-stranded RNA viruses (Rice et al., 1985; Rice et al., 1986). The N terminus of NS5 is homologous with several methyltransferase proteins and this domain may be involved in the methylation of the 5' cap.

The genomic organization of flaviviruses is presented in Fig 1.3. The complete nucleotide sequences of flaviviruses from several serological complexes are currently available (Chambers et al., 1990). In a more recent phylogenic study, Kuno et al., (1998) established a genetic relationship among the viruses of the genus *Flavivirus*. The ranges in pairwise amino acid sequence identities on NS5 gene were 75 to 86% in DEN complex viruses, 83 to 96% in JE complex viruses and 72% between YF and Banzi viruses (Kuno et al., 1998).

1.3 Flaviviral replication cycle

Viruses are obligate intracellular pathogens that depend on host cell molecules to complete their replication cycles. Most flaviviruses require an arthropod vector (mosquitoes or ticks) for replication and a vertebrate host for their natural transmission cycle. Flavivirus replication studies are done in cell cultures derived from mammalian, avian and arthropod sources (Rice, 1996). Virus replication in arthropod cell lines is mostly noncytocidal, whereas replication in vertebrate cell lines generally produces pronounced cytopathic effects. Those observations are in accordance with the non-pathological effects that have generally been described in arthropods, and with the clinical disease that is often present in infected vertebrates. A schematic representation of flaviviral replication is shown in Fig. 1.4.

There is evidence that the infection of cells is initiated by the entrance of the virus via receptor-mediated endocytosis (Rice, 1996; Heinz and Allison, 2000). It has been shown that a slightly acidic pH triggers fusion between the viral and the endosomal membranes, which, in turn, produces a conformational change in the viral E protein. This change is followed by an uncoating of the nucleocapsid in the cytoplasm (which is the site of flavivirus replication in the infected cell) to initiate translation and RNA replication (Rice, 1996; Heinz and Allison, 2000). Lee and Lobigs (Lee and Lobigs, 2000) demonstrated that a hydrophilic region (FG loop) of the E protein has a functional role in virus entry; which suggests that flaviviruses can enter cells by attaching themselves to glycosaminoglycans.

Antibody-dependent enhancement (ADE) has been described as an alternative entry route of flaviviruses into cells (Hawkes, 1964; Halstead, 1982). ADE involves the presence of subneutralizing concentrations of antibody that is bound to virus and which mediates the uptake of virus/Ab complex into Fc receptor-bearing cells. This mode of entry seems to be important in secondary flavivirus infection, in particular with dengue virus. For DEN and YF viruses, the initial interaction site of the virus with the host is heparan sulfate, which allows the virus to bind to the surface of the cell and infect the host (Germi et al., 2002). In this process virions localize to clathrin-coated pits on the cell surface and are rapidly taken up into coated vesicles. Lee and Lobigs (Lee and Lobigs, 2000) found evidence that cell-surface glycosamminoglycans are involved in attachment of MVE, with the degree of involvement depending on the dose and cell type that is used.

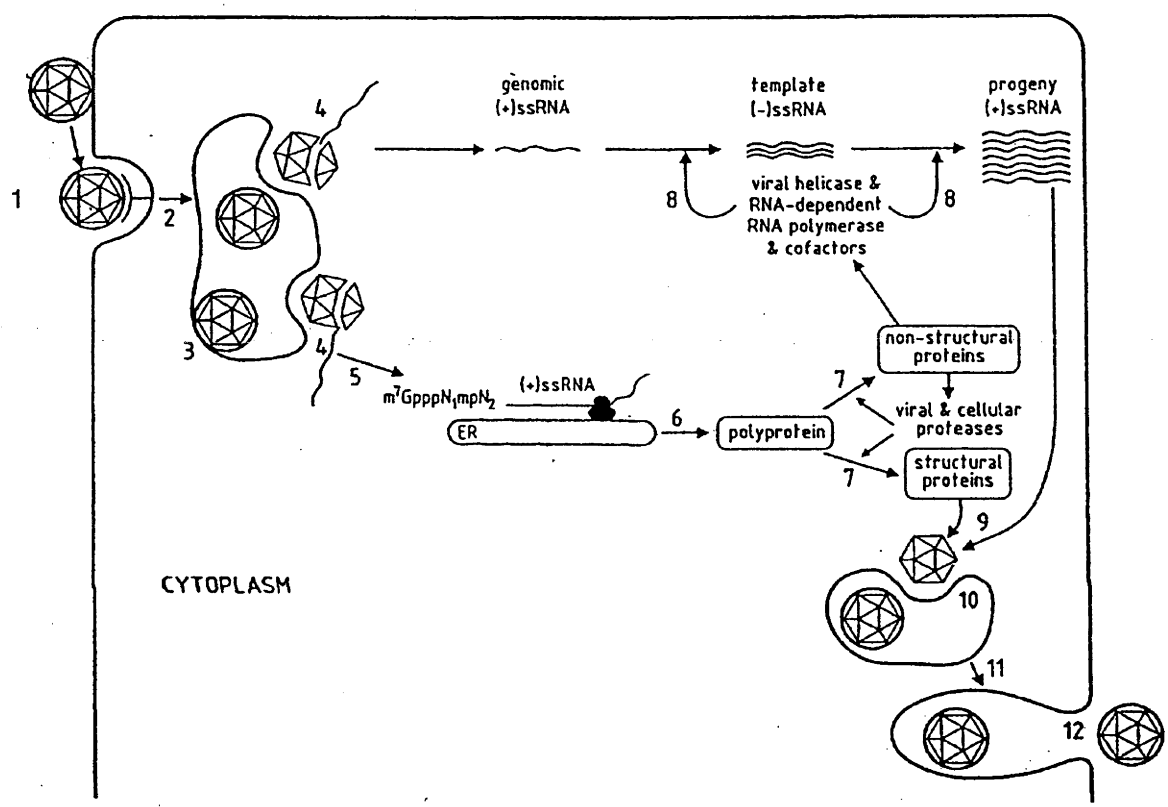

Fig. 1.4 Replicative cycle of flaviviruses. 1, adsorption; 2, receptor-mediated endocytosis; 3, low-pH fusion in lysosomes; 4, uncoating; 5, cap-mediated initiation of translation; 6, translation of the viral RNA into viral precursor polyprotein; 7, cotranslationally and posttranslational proteolytic processing of the viral polyprotein by cellular and viral proteases; 8, membrane-associated synthesis of template minus-strand RNA and progeny plus-strand RNA; 9, assembly of the nucleocapsid; 10, budding of virions into the endoplasmic reticulum; 11, transport and maturation of virions in the endoplasmic reticulum and the Golgi complex; 12, vesicle fusion and release of mature virions. ss, single stranded. Modified from (Leyssen et al., 2000).

The viral polyprotein is processed cotranslationally and posttranslationally into individual and functional viral proteins. This processing is carried out by cellular and viral proteases. The RNA-dependent RNA polymerase, associated with cofactors, produces minus- and plus-stranded RNA. After replication, the viral genome is encapsidated by association with C protein and acquires an envelope to become a mature virus (Leyssen et al., 2000). The virus particles, packed within secretory granules, are released at the cell surface by exocytosis. Virus is thus released via the same pathway as the cell's normal secretory products (Monath, 1986).

1.4 Pathogenesis of flaviviral encephalitis

The flaviviruses that typically produce meningoencephalitis in humans are JEV, MVE, SLE, and TBE. Although WNV can also produce encephalitis in humans, its most characteristic symptoms resemble those of dengue and polio. Another characteristic of WNV and TBE, which differentiates them from JEV and MVE, is that they can frequently be isolated from the blood of patients during the acute phase. Other encephalitic flaviviruses do not produce detectable viremia (MacDonald, 1952; Huang, 1963; Monath, 1996; Lee and Lobigs, 2002; Diamond et al., 2003b; Lobigs et al., 2003b).

Experiments with mice provide the greatest body of information about flavivirus pathogenesis. Mice are suitable to reproduce pathogenesis of flavivirus encephalitis, but not to reproduce other syndromes that are associated with human flavivirus infection, such as hepatitis, arthralgia, rash, and hemorrhagic fever. Viruses (including DEN and YF) that produce these other syndromes in humans and primates cause encephalitic

infections in laboratory rodents. The universal neurotropism of flaviviruses in rodents, and even in arthropod vectors (in which brain and ganglia are major sites of replication), may indicate that there has been an evolutionary conservation of the viral polypeptide structures that are involved in receptor interactions and of cell membrane molecules which subserve the virus–receptor interactions (Monath, 1996).

The pattern of pathogenesis of flaviviral encephalitis depends upon the extent of the virus replication and on the degree of the inflammatory response in the host. Infection with encephalitic flaviviruses can thus have one of three general patterns of pathogenesis: 1) early viremia and extensive extraneural replication, which usually results in fatal encephalitis; 2) low viremia, late initiation of brain infection and clearance with minimal destructive pathology, which may lead to subclinical encephalitis; and 3) trace viremia and no neuroinvasion, which results invariably in an unapparent infection (Monath, 1996). The mechanisms involved in the definition of each of the above patterns of pathogenesis have not yet been fully defined. Major obstacles in attempts to define the role of humoral and cellular immune responses during flaviviral infections have been, like in humans, that mice show individual variability in susceptibility to infection and the lack of an effective immunocompetent animal model (Hall et al., 1996).

There are only a few reports on the pathogenesis of JEV in adult mice (Mathur et al., 1983; Lange and Sedmak, 1991; Lad et al., 1993) and many of its aspects still need to be elucidated. JEV is closely related to MVE and both would normally be regarded as sufficiently similar to allow findings with regard to the pathogenesis of one to help in the understanding of the pathogenesis of the other. While caution should be exercised when generalizing on the role of the immune response to JEV and MVE infections, there are grounds to suggest that MVE is a potential model for JEV.

1.4.1 Factors influencing pathogenesis

In general, the spectrum of neurotropic flavivirus infection depends on the virus–host pairing, the virus dose, and on other factors that influence the host response. Among the host factors that influence pathogenesis, the most important factors are: age, genetic susceptibility, and pre-existing infection or immunity to heterologous agents (Monath, 1996). The level of neurovirulence and neuroinvasiveness of a particular virus strain also influences infection (Monath, 1996).

Infant mice are highly susceptible to flavivirus infection by all routes of inoculation. In humans, a bimodal (young–elderly) pattern of susceptibility is observed with JEV and MVE infections. In the case of SLE and WNV, however, susceptibility to encephalitis increases with advancing age, with the elderly being most severely affected (Monath, 1996). There are no known reports that there exists a model in laboratory animals that could represent the increased susceptibility to encephalitic flavivirus in old people (Monath, 1986). Susceptibility in the young seems to be related to immune immaturity (Ogata et al., 1991), and to the fact that the blood–brain barrier (BBB) is not yet fully established. Factors that may be important for the increased susceptibility seen in the old may include age-related waning of immunological responsiveness, and the presence of underlying diseases that impair the immune function or reduce the effectiveness of the BBB (Monath, 1996).

Sex differences in susceptibility of humans to flavivirus infections have not been demonstrated. Possible exceptions include MVE, which seems to occur more frequently in men than in women (although this may be related to the increased exposure of men to MVE-infected mosquitoes due to their more frequent outdoor activities), and DHF, which seems to affect female children preferentially (Monath, 1996).

Genetic determinants play a central role in the pathogenesis of flavivirus infections. Miura et al. (1990) investigated resistance to JEV using inbred C57Bl/6 and C3H/He mouse strains. C57Bl/6 mice immunized with JEV were resistant, whereas most C3H/He mice treated the same manner died. Genetic resistance to flavivirus is due to different genes. However, the most adequately studied allele that confers flavivirus resistance is the single autosomal, dominant allele *Flv* (Brinton and Perelygin, 2003).

1.4.2 Extraneural infection

Extraneural flaviviral replication generally occurs in mice that are younger than 3 weeks (Monath, 1996). After inoculation into the skin, the virus replicates in local tissues, spreads to regional lymph nodes, and is released into the bloodstream. This primary viremia seeds extraneural tissues, which, in turn, support further viral replication and act as a source for the release of virus into the circulation (Monath, 1996). Primary extraneural sites for flaviviral replication include connective tissue, skeletal and smooth muscle (myocardium), lymphoreticular tissues, and endocrine and exocrine glands (Monath, 1996).

In studies of virus distribution in MVE-infected mice, MacDonald (MacDonald, 1952) showed that intramuscular inoculation of the highly passaged prototype virus MVE-1–51 (French, 1952) into 1-week-old outbred mice resulted in the simultaneous appearance of infectious virus at the inoculation site and in the bloodstream between 2 and 4 days postinfection (pi), followed by infection of the central nervous system (CNS) between 3 and 5 days pi. In 3-week-old Swiss mice, infected with a highly neuroinvasive MVE strain (BH3479), virus was first detected in lymph nodes that drain the inoculated footpad at 24 h pi, in serum between 36 and 72 h pi, and in the CNS at day 4 pi (McMinn et al., 1996). MVE BH3479 appeared to enter the CNS via the olfactory lobes, since it spread in a rostral to caudal direction over 3–4 days. The fact

that MVE was found simultaneously in the inoculation site and in the bloodstream during MacDonald's work (MacDonald, 1952), and that it appeared first in the olfactory bulb before entering the brain in McMinn's work (McMinn et al., 1996), suggests that MVE could use the hematogenous and/or neuronal routes of infection.

1.4.3 Neuroinvasion and pathological changes in the CNS

The entry of neurotropic viruses into the CNS is a central issue in the pathogenesis of these viruses, since the involvement of the brain appears essential for the virus to cause morbidity and mortality in the clinical hosts (Mathur et al., 1982; Monath, 1996).

Animal studies indicate that flaviviruses cross the blood–brain barrier (BBB) and gain entry into the CNS via two routes: 1) The hematogenous route, where passive diffusion or transcytosis of virus through endothelial cells of cerebral capillaries results in budding of virus into the brain parenchyma (Liou and Hsu, 1998). This route of neuroinvasion probably occurs in mice of three weeks of age or younger, which sustain high viremia. However, there are doubts as to whether this route is relevant in clinically important hosts (humans and horses). 2) Because viremia in humans and horses is of brief duration and low magnitude, the neuronal route, where virus infects olfactory neurons at the olfactory epithelium (unprotected by the BBB) may be an alternative pathway to gain access to the CNS (Monath et al., 1983; Monath, 1986; Kobiler et al., 1989; Hase et al., 1990; Lustig, 1992).

Generally, the distribution of flavivirus antigen in the brains of laboratory rodents is similar to that in the brains of humans. The most severely affected areas in the CNS are the thalamus, caudate nucleus, putamen, and substantia nigra (Ogata et al., 1991). Pathological changes include neuronal and glial damage by direct viral injury,

enhanced lesions by inflammatory responses, cellular nodule formation composed of activated microglia and mononuclear cells (Koprowski et al., 1993; Monath, 1996), and cerebral interstitial edema (Monath, 1996; Wasay et al., 2000). Neuronal and glial damage is characterized by central chromatolysis, cytoplasmic eosinophilia, cell shrinkage and neuronophagia (Monath, 1996). Studies by Despres et al. (1996) have shown that DEN virus and JEV induce apoptosis in infected neuroblastoma cells *in vitro*, suggesting that this may be a mechanism by which flavivirus infection may damage neurons within the CNS.

Although flaviviruses are generally considered to be cytopathic viruses, a study by Andrews showed that MVE infection of neurons does not result in cell lysis, despite the detection of high levels of viral replication in the CNS (Andrews et al., 1999). Andrews' findings in mice are consistent with those of Johnson et al. (1985), who studied fatal encephalitis in children. In Johnson et al.'s studies, encephalitis in infants results mainly from infection in neurons, although there is little neuronal lysis. Thus, direct virus-induced cell injury may not be an important mechanism in the pathogenesis of certain flavivirus-mediated encephalitis.

The occurrence of encephalitis may be explained by functional alterations in infected neurons rather than by cell destruction or by inflammatory responses that may enhance lesions and accelerate death (Monath, 1996). Inflammation of the brain parenchyma includes perivascular infiltration of small vessels with lymphocytes, plasma cells, and macrophages (Johnson et al., 1985; Monath, 1996). In Johnson et al.'s study of the composition of the mononuclear inflammatory response in children that died of JE, quantitation of perivascular inflammatory responses showed a preponderance of cytotoxic T cells of 7 to 30% (Johnson et al., 1985). Neutrophil recruitment into the CNS in response to infection, trauma and infarction, has also been shown to have a

deleterious effect on brain function. This may be due to the release of toxic inflammatory mediators or due to a secondary effect of the neutrophil-mediated breakdown of the BBB, which results in an altered CNS homeostasis (Andrews et al., 1999).

Many of the clinical manifestations of encephalitis appear to be due to functional alterations of certain areas of the brain. The predominant involvement of the thalamus and brain stem is consistent with frequent tremors that are seen during the acute disease. The infection and destruction of neurons, together with an increase in intracranial pressure, explains the deep coma and respiratory arrest. The Purkinje cell loss presumably results from hyperthermia or anoxia (Johnson et al., 1985), while spasticity is associated with damage to the corpus striatum, and flaccid paralysis is associated with the infection of the spinal cord central horn motor neurons (McMinn et al., 1996).

Persistence of flavivirus in the host may result in a chronic illness (Monath, 1996). For example, Ravi et al., (1993) observed that there was persistence of JEV in the human CNS in 5% of their studied cases; and Mathur found that in congenitally infected Swiss mice (after their mothers had been given JEV intraperitoneally during pregnancy) there was latent JEV infection, and that 2.7% of the mice showed persistence of infectious virus until they were 35 days old (Mathur et al., 1986). Monath also demonstrated that JEV persists for prolonged periods in lymph nodes of athymic nude mice in the absence of clinical signs (Monath, 1986), and that residual neurological deficits, electroencephalitic changes and psychiatric disturbances in humans persist in more than half of the survivors after they recover from acute encephalitis (Monath, 1986). TBE has also been shown to persist in human kidney (RH) cells for at least 60 days, probably due to the production of modified NS1 protein oligomers that can induce slow virus replication (Bugrysheva et al., 2001).

1.5 The innate immune response

Given that some viruses are capable of completing an infectious cycle in just a few hours, and that specific immune mechanisms require 3 to 4 days to reach even minimally effective levels, the potential for damage is enormous (Yewdell, 1997). In the case of flaviviruses, progeny first becomes visible in infected cells by electron microscopy 8 to 12 hours after infection (Westaway et al., 1985). Thus, in the initial encounter with a virus the innate immune response plays a crucial holding action in preventing massive tissue destruction and virus dissemination (Yewdell, 1997).

1.5.1 Cytokines and other antiviral soluble factors

Cytokines are a group of non-enzymatic protein hormones whose actions affect a variety of functions, including cellular metabolism, growth and differentiation, immune function, and tumor development (reviewed in (Kelso, 1998)). Cytokines are released by many types of cells in response to being infected, or in recognition of infection, and their functions are mainly immunoregulatory (Harty et al., 2000). Flavivirus infection can be inhibited by cytokines (mainly interferons α and ß) in the absence of specific antibodies (Hasegawa et al., 1990; Goodbourn et al., 2000; Biron, 2001; Lobigs et al., 2003b).

The interferons (IFNs) are a family of cytokines that are essential to the host antiviral response, which is normally induced within hours of systemic viral infection and decreases with resolution of the infectious state (Slifka et al., 1999). The IFNs comprise two major subgroups (based on their properties and cellular receptors), termed type I (IFN-α and IFN-ß) and type II (IFN-γ).

IFN-α is produced predominantly by leukocytes (in particular dendritic cells and macrophages), whereas IFN-ß is produced by a large variety of cells, including fibroblasts, epithelial cells, and macrophages. Type I IFNs share a common receptor and have closely overlapping biological actions.

Expression of type I IFNs can be induced by many factors, but is particularly induced by infection with viruses (Slifka et al., 1999; Harty et al., 2000). Besides induction of important antiviral activities in infected cells, type I IFNs also activate NK cells and enhance the antigen processing and presenting capacity of antigen-presenting cells (APCs) (Yewdell, 1997). Expression of the type I IFN genes may be induced in the CNS in different viral infections (e.g. lymphocytic choriomeningitis virus (LCMV) infection in mice). Glial cells (astrocytes and microglia) have been shown to produce type I IFNs in vitro, which suggests that these cells may provide a local source of IFN that is produced to defend the brain against a viral insult. Type I IFNs may be important contributors to the recovery from encephalitic flavivirus infection because their inducers have been shown to have a prophylactic and therapeutic effect (Haahr, 1971; Vargin et al., 1977; Taylor et al., 1980), and because recombinant IFNs modulate nonspecific immunity and are effective antiviral agents *in vivo* (Pinto et al., 1988; Brooks and Phillpotts, 1999). However, there is also evidence to suggest that the type I IFNs may be toxic to the brain (Akwa et al., 1998).

Type II IFN or IFN-γ possesses several characteristics that are unique and relate particularly to its immunoregulatory functions, but also shares many characteristics (overlapping and synergic actions) with type I IFNs (Akwa et al., 1998). Activated T lymphocytes and natural killer (NK) cells produce type II IFN, which binds to a unique receptor (Yewdell, 1997; Harty et al., 2000). Akwa et al. (1998) generated transgenic mice that produce IFN-γ chronically in astrocytes, which protects them

against lethal neurotropic viral infection (e.g. LCMV) but also induces inflammation and neurodegeneration. Mice with targeted disruption of the IFN-γ gene are not significantly different from wild-type mice in their susceptibility to DEN (Johnson and Roehrig, 1999), YF (Liu and Chambers, 2001) or MVE (Lobigs et al., 2003b). It may be that the potentially deleterious inflammatory response, resulting from the production of IFN-γ during DEN and YF infections, is compensated by the IFN-γ antiviral effect.

Tumor necrosis factor alpha (TNF-α) is a multifunctional pro-inflammatory cytokine, which has a pivotal regulatory function during infection and inflammation and is also implicated in the pathogenesis of autoimmune diseases (Stalder et al., 1998). The sources of TNF-α may be infiltrating neutrophils, macrophages and T cells. Astrocytes, microglia and possibly neurons may also produce TNF-α locally in the CNS (Stalder et al., 1998). TNF-α is activated in the CNS during various insults, and has both detrimental and protective functions. For example, knockout mice that are deficient in the TNF genes show an increase in experimental allergic encephalitis (EAE), but are resistant to cerebral malaria (Canque, 1992; Lucas et al., 1997). Stalder et al. (1998) showed that persistent TNF-α expression in astrocytes is sufficient to induce a chronic inflammatory encephalopathy in which macrophage/microglial cells play a central role in the mediation of injury. MVE induces an inflammatory response in the CNS of weanling mice five days pi, preceded by an increased expression of TNF (Andrews et al., 1999). A possible explanation for the TNF-α-induced immunopathology that occurs during MVE infection of mice may be that resident CNS cells stimulate the expression of the neutrophil chemo-attractant N51/KC (murine homologue of human interleukin-8), and that this results in neutrophils infiltrating across the cerebral endothelium into the CNS parenchyma. Chemotaxis of neutrophils across the cerebral endothelium leads to disturbance of the integrity of the BBB. Neutrophils in the CNS

parenchyma affect the release of inflammatory mediators such as nitric oxide (NO), and disturb CNS homeostasis and neuronal function (Andrews et al., 1999).

Nitric oxide is a potent microbicidal and tumoricidal agent, and a mediator of antiviral defense. NO is produced by neutrophils and macrophages. As a disregulated production of NO may lead to extensive tissue damage, it is important that the production of this mediator remains highly regulated (Kreil and Eibl, 1996). However, the antiviral activity of NO *in vitro* may be confined to certain viruses, while its effect on other viruses may be neutral (Kreil and Eibl, 1996; Andrews et al., 1999). Viral infections may alter the regulation of NO by either priming for, or down-modulating NO production. Because NO is generally considered to be an antiviral agent, the down-modulation of NO production during viral infection would be opposite to antiviral protection (Kreil and Eibl, 1996). The antiflaviviral activity of NO has been demonstrated for JEV (Lin, 1997; Saxena et al., 2000), several poxviruses and herpes simplex virus infections (Kreil and Eibl, 1996). However, Kreil and Eibl (1996) showed that high levels of NO production do not have an inhibitory influence on TBE replication *in vitro*, and that when aminoguanidine, an inhibitor of NO production, is administered *in vivo* to TBE-infected mice, it significantly increases their survival time. This is evidence that NO production may even contribute to pathogenesis of viral infection *in vivo* (Kreil and Eibl, 1996). Andrews et al. (1999) showed that NO also contributes to the disturbance of CNS homeostasis and to the neural function of 3-week-old mice infected with MVE. The combined effect of NO and TNF-α could affect the integrity of the BBB of MVE-infected mice (Andrews et al., 1999).

The complement system plays different roles during the immune response, including immunoregulation, phagocytosis, membrane lysis, inflammation, and antibody-dependent enhancement (ADE) (Yewdell, 1997).

1.5.2 The cellular innate antiviral responses

Different cell types have been implicated in the early antiviral responses: 1) phagocytic cells (macrophages and neutrophils) which engulf and destroy viruses, 2) cells which release cytokines with antiviral or immunoregulatory activities (macrophages, neutrophils, mast cells, basophiles), 3) natural killer (NK) cells which recognize virus-infected cells based on alterations that are common to many virus infections and that either destroy the infected cells or release cytokines (Yewdell, 1997), and 4) γδ T cells which reduce the susceptibility of mice to WNV infection (primarily due to the IFN-γ- production of γδ T cells) (Wang et al., 2003). Presumably, these host defense mechanisms (together with IgM antibodies) are often sufficient to limit infection with flaviviral strains of marginal neuroinvasiveness (Monath, 1986).

Macrophages play an important role in the defense against most infections (Blanden, 1982). During the early stages (one to seven days) of viral infection, virus clearance from blood, and host resistance conferred by macrophages, is due to virocidic properties, whereby highly cytotoxic macrophages appear in the peritoneum and lymph nodes prior to detectable specific antibodies (Rodda and White, 1976; Khozinsky et al., 1985; Hill, 1993; Yewdell, 1997). Macrophages that are obtained late after infection with flaviviruses mediate specific antibody-dependent cell cytotoxicity (Hill, 1993). Macrophages also function as major antigen-presenting cells (APCs) (Hill, 1993). The complexity of flavivirus–macrophage interaction indicates that permissiveness to virus growth varies with the physiological state of macrophages, age and strain of mouse, and receptor pathways of virus entry (Cardosa et al., 1986). Infection with TBE, combined with temporary inhibition of phagocytosis by macrophages, results in an increased lethality (Khozinsky et al., 1985). Induction of peritoneal macrophages by concavalin A mediates a protective effect against JEV infection (Hill, 1993). In primary flavivirus

infection, macrophages have been shown to have an effect on pathogenesis, although information is still limited (Monath, 1996). Interactions between macrophages, viruses and antiviral antisera may paradoxically result in an increase in viral infectivity, which could be produced by ADE (Peiris and Porterfield, 1979).

Neutrophils are mediators in innate immune reactions. However, work by Andrews et al. (1999) has implicated neutrophils in the pathogenesis of MVE. They found that neutrophils predominated in a mixed-cell inflammatory infiltrate from brains of MVE-infected mice, and that their appearance correlated with the onset of lethal encephalitis between 5 and 6 days pi. Depletion of neutrophils resulted in prolonged survival and decreased mortality. The prolonged survival of neutropenic MVE-infected mice was not due to reduced viral neuroinvasion, because virus entered the CNS at 5 days pi, in both neutrophil-depleted and mock-depleted mice, and because virus titers in the CNS of both groups were almost identical. MVE-encephalitis was not due to induction of apoptosis of infected neurons, since < 0.1% of infected neurons showed evidence of apoptosis. Encephalitis was presumably due, in addition to infiltration of neutrophils in perivascular regions and CNS, to the consequent increased expression of TNF-α and induced nitric oxide sythase activity (Andrews et al., 1999).

Natural killer (NK) cells are a lineage of lymphocytes that do not express antigen-specific cell surface receptors (Renard et al., 1997). They can lyse some tumors and virus-infected cells and can mediate acute rejection of bone-marrow cell grafts (George et al., 1997). NK cells recognize and eliminate potentially pathological cells that fail to express, or have reduced, self – major histocompatibility complex (MHC) class I molecules (Ljunggren and Karre, 1990). NK cells can directly interfere with viral replication by releasing cytokines (predominantly IFN-γ and-β) with antiviral activity, or by lysing virus-infected cells. NK cells can directly lyse cells in one of two ways: 1)

by antibody-dependent cell-mediated cytotoxicity (ADCC); or 2) by cell-mediated cytotoxicity of virus-infected cells, where lysis is usually mediated by the perforin/granzyme mechanism also used by T cells (detailed in section 1.6.2). Viral infections in general elicit pronounced NK cell activity two to three days post-infection (pi) (Hill, 1993). However, the typical upregulation of MHC class I expression observed on cells infected with flaviviruses such as WNV, YF, DEN, MVE, KUN and JEV may be responsible for the greatly impaired NK-cell lysis of flavivirus-infected target cells (King, 1988; King et al., 1989; Liu et al., 1989b; Mullbacher, 1995; Lobigs et al., 1996; Momburg, 2001). Hill and colleagues (Hill, 1993) investigated the induction of NK cells after infection with WNV in mice, and their ability to lyse WNV-infected target cells. They found that the NK response was only marginally elevated in comparison to that in the spleen of infected mice from control animals, suggesting that the increased class I MHC expression on the surface of infected cells may have impaired induction and function of NK cells (Hill, 1993; Momburg, 2001). Liu et al. (1989a) demonstrated that there is induction of NK-like cells in the CNS after intracranial (ic) infection with WNV in mice, which suggests that there is NK activation at the site of flavivirus replication *in vivo*. However, given that virus will leak from the ic inoculation site into extraneural sites, it is not clear if the induction of the NK cell response occurred in the CNS from a peripheral reaction. Liu et al. recorded an additional upregulation of MHC class I gene expression, which was most likely caused by productive WNV infection of astrocytes. However, even prior to finding detectable levels of class I MHC protein on astrocytes, these cells were still refractory to virus-induced NK cell lysis (Liu and Mullbacher, 1988). Consequently, cytokines released from flavivirus-induced NK cells may have had a direct or indirect influence on the outcome of such infections (Hill, 1993).

1.6 The adaptive immune response

The immune response to neurotropic viruses competes in a 'race' with the rate and extent of virus replication, resulting in neuroinvasion and encephalitis when the development of immune responses has not been effective (McMinn et al., 1996). The components of the adaptive immune system consist of T and B cells. Both types of immune cells express, in a clonally restricted manner, receptors on their surface that interact specifically with a limited set of related antigens (Yewdell, 1997).

There are two dominant types of peripheral T cells, $CD8^+$ T cells and $CD4^+$ T cells, and both express a surface antigen T-cell receptor (TCR). Antigen-specific T-cell responses are induced by the binding of the TCR with MHC-peptide complex, with short (8–10) or somewhat longer (10–34) peptides. The short peptides are presented by MHC class I molecules, and the longer peptides are presented by class II molecules (Appella, 1995; Harty et al., 2000). $CD8^+$ T cells predominantly recognize cells that present fragments of endogenously derived peptides presented via MHC class I molecules. Presentation of antigens to $CD4^+$ T cells occurs after endocytosis or phagocytosis of an Ag, which is processed in a lysosomal compartment to peptides and presented by MHC class II molecules. Antigen-presenting cells (APCs) express great concentrations of classes I and II MHCs on their surface, together with the foreign antigen. The professional APCs are dendritic cells, macrophages and B lymphocytes that present antigens to the $CD4^+$ T cells (Th, or helper cells). Thus, there is a synergistic Th and B cell effect in both directions: B cells are activated to proliferate and to produce antibodies, while Th cells are stimulated by the antigen that is taken up, processed and presented on the surface of B cells (Hill, 1993).

Antibodies produced by B cells are immunoglobulins (Ig) that bind antigen molecules. B cells first produce a membrane-bound form of Ig, the B cell receptor

(BCR), as part of B cell differentiation. Each B cell expresses one Ig, but the population of B cells in each individual displays a wide variety of antigen specificity, with each cell expressing Ig on its surface as part of the B cell receptor complex. There are five classes of human Igs: IgM, IgA, IgD, IgE, and IgG. Human patients with acute encephalitis have mainly virus-specific IgM that peaks at day 7 after hospitalization (Burke et al., 1985b). Relatively little is known about the immune responses during human flaviviral encephalitis, and most of the existing knowledge has been derived from experiments in mice (Monath, 1986).

1.6.1 The humoral immune response

Antibody production has been demonstrated to be the main mechanism of protection in several flaviviral studies in mice (Monath, 1986). In mice inoculated with encephalitic flaviviruses, neutralizing Ab appears between 4–6 days postinoculation (Webb et al., 1968; Monath and Borden, 1971; Camenga et al., 1974), with IgM predominating in the early phase of infection (Webb et al., 1968; Monath and Borden, 1971). The antibody production to some flaviviruses (e.g. JEV) is thymus-dependent and appears to be fully dependent on helper T cells (Mathur et al., 1982; Lad et al., 1993). Passively administered antibody against flaviviruses protects the recipient mice against infection, even if neuroinvasion has already occurred (Mathur et al., 1982; Monath, 1986, 1996). The protective role of B cells is further demonstrated when mice that are defective in B cells or $CD4^+$ T cells do not survive a lethal JEV challenge (Pan, 2001).

IgM antibodies predominate in the early phase of flaviviral infection of mice and have a half-life of about 4 days (Mathur et al., 1982; Monath, 1986). McMinn et al.

(McMinn et al., 1996) found that there is a clear association between the time of appearance of anti-MVE IgM and the first significant fall in the level of viremia (between 3 and 4 days pi). Protection is abrogated in JEV-infected mice by pretreatment of the early serum with 2-mercaptoethanol to dissociate IgM (Mathur et al., 1982). Boosting and sustaining the production of IgM has been demonstrated to be essential for protection and may be considered as the main host defense mechanism against encephalitic flaviviruses (Mathur et al., 1982).

Monath (1986) found that IgM and IgG may co-circulate in blood during a brief period of immune clearance. In experiments with MVE in mice of 3 weeks of age, anti-MVE IgG is first detected at 6 days pi and peaks between 9 and 14 days pi (McMinn et al., 1996). The anti-MVE IgG response appears to be associated with clearance of infectious virus from the CNS (McMinn et al., 1996). In humans, studies of systemic infection and local CNS antibody response to JEV have shown that there is a strong correlation between survival and an early, and quantitatively strong, antibody response (Burke et al., 1985a). Monath (1986) also found that patients who survive JEV have serum IgG antibody during the first five days of admission to hospital, whereas fatal cases only become seropositive later.

Antibodies have the ability to signal the complement system, which coats the antigen, thus rendering it prone to phagocytosis. Although blocking the attachment of flavivirus to target cells is by far the most common means of antibody neutralization (Cardosa, 1998), some of the E protein epitopes are abolished by the conformational transitions that take place at low pH. Such conformational transitions are postulated to be important in the fusion event, which is necessary for the establishment of infection. Prevention of pH-dependent fusion is an alternate mechanism to neutralize flavivirus

infection. Thus, blocking of virus attachment to the target cell is not the only mechanism of neutralization of flaviviruses (Vorovich et al., 1991).

Antibody-dependent enhancement (ADE) of viral infectivity occurs when cells that bear Fc receptors on their surface are infected with virus that has been in contact with antiviral antibody that is insufficient to cause viral neutralization. ADE of infection with flaviviruses has been demonstrated *in vitro* for DEN (Peiris and Porterfield, 1979; Legrand et al., 1986; Mady et al., 1991), WNV (Peiris and Porterfield, 1979), TBE (Phillpotts, 1985, 1987; Kopecky et al., 1991), JEV (Phillpotts, 1985), YF (Phillpotts, 1985); and *in vivo* for YF (Barrett and Gould, 1986), DEN (Morens, 1994) and JEV (Gould and Buckley, 1989). *In vivo* enhancement of TBE infection has not been demonstrated, even though ADE has been demonstrated *in vitro* using the same combination of TBE antibodies and virus. Thus, ADE of viral infection *in vitro* does not always predict enhancement *in vivo* (Kreil and Eibl, 1997). It has been suggested that ADE represents immunopathological processes that occur *in vivo*; mainly for DHF/DSS, and that ADE may be a limitation for vaccine development against DEN (Morens, 1994; Cardosa, 1998).

Mouse monoclonal antibodies have been described as being able to neutralize flaviviruses (Cardosa, 1998). The humoral immune response is predominantly directed against the viral envelope (E) protein and a cell surface localized non-structural protein (NS1) (Monath, 1986). Antibodies directed against NS1 on the surface of infected cells appear relatively late and probably play an important role in viral clearance (Monath, 1986). Schlesinger and co-workers (Schlesinger et al., 1985) first showed that immunization with purified NS1 of YF and passive transfer of monoclonal antibody to NS1 both result in protection against YF17D-induced encephalitis. DNA immunization of mice with JEV NS1 gene also results in protective immunity (Lin et al., 1998).

Much interest has been centered on the identification of protective B- and T-cell antigens, in order to develop anti-flavivirus vaccines, particularly against dengue (Brandt, 1988), and to improve the existing JE vaccine. When certain flaviviral antigens are encoded in the host through recombinant DNA technology, humoral as well as cellular responses against flaviviruses are observed (Monath, 1986; Klinman et al., 1999). Pan (2001) demonstrated the importance of B cells in the protection from lethal virus challenge using DNA-vaccination against JEV E protein. Colombage et al. (1998) investigated the immunogenicity and protective efficacy of DNA-based vaccination with plasmids that encoded the membrane proteins prM and E of MVE. They elicited long-lived virus-neutralizing antibody responses in mice by intradermal delivery of DNA, which encodes the prM and E proteins. This resulted in protection, although mice were challenged with high titers of MVE inoculum. Induced MVE-specific IgG1 antibodies were prevalent after intradermal DNA vaccination, but not after the whole virus was injected by the same route (Colombage et al., 1998). This result represents interesting possibilities for DNA vaccination.

1.6.2 The cellular adaptive immune response

Cellular-specific adaptive immune mechanisms are basically executed by T cells. These are generally important for the recovery from intracellular pathogens such as viruses. Both $CD8^+$ MHC class I – restricted and $CD4^+$ MHC class II – restricted T cells may be responsible for either: 1) Viral clearance and recovery. Viral clearance may be facilitated in the case where target cells are lysed before the assembly of progeny virus. 2) Additional dissemination of the virus. This may be facilitated if target cells are lysed after the assembly of a significant amount of progeny virus.

3) Immunopathology (Hill, 1993). Virus-specific T lymphocytes have been shown to be critical for clearance of poxvirus (Blanden, 1974; Welsh, 1981; Karupiah et al., 1990; van Binnendijk et al., 1990; Karupiah et al., 1996) and influenza virus (Topham et al., 1996; Topham et al., 1997; Cerwenka et al., 1999; Riberdy et al., 2000). However, T lymphocytes can also exacerbate tissue injury and induce disease. This has been demonstrated in mice infected with LCMV, respiratory syncytial virus, and other viruses (Rothman and Ennis, 1999). T-lymphocyte-mediated immunopathology may be relevant in the induction of DHF during secondary dengue virus infections (Rothman and Ennis, 1999). Activation of DEN virus-specific T lymphocytes during interactions with dengue-virus infected monocytes may result in an increased capillary permeability through cytokine production and cytolytic mechanisms of dengue virus-specific T cells (Rothman and Ennis, 1999). Certain types of T cells play different roles during infections, i.e. MHC class I – restricted $CD8^+$ T cells have been shown not to be required for resistance to vaccinia virus, vesicular stomatitis virus or Semliki Forest virus; while antibodies, MHC class II – restricted $CD4^+$ T cells and cytokines contribute significantly to clearance of these viruses (Kagi et al., 1995).

During flavivirus infections, the participation of T cells to the course or outcome of the infection is influenced by the virus strain (Monath, 1986). Mild flaviviral replication can be aborted by the non-specific defense mechanisms, while T cells, together with other responses, are required for clearance of more virulent viral strains (Monath, 1986). Flavivirus-induced upregulation of MHC class I molecules on infected cells leads to a more efficient lysis of infected cells by cytotoxic T (Tc) cells, although the susceptibly to NK-cell-mediated lysis may be reduced (Liu et al., 1989b; Lobigs et al., 1996).

Some researchers have postulated that T cells play a role in the control of primary flaviviral infection, based on reconstitution experiments and immunosuppression, T-cell cytotoxicity, measurement of delayed-type hypersensivity (DTH) responses and leukocyte migration inhibition (LMI) (Mathur et al., 1983; Monath, 1986). As most of these studies are done *in vitro*, the question of whether an equivalent situation occurs *in vivo* is raised. New methods for the study of Tc cells, such as staining with tetrameric MHC class I peptide complexes, intracellular cytokine staining and ELISPOT analysis have been used for the understanding of the T-cell response to infection (Harty et al., 2000).

During flavivirus infections of mice, *in vitro* T-cell immunity has been demonstrated through cytotoxic assays (reviewed in (Monath, 1986)). Humoral as well as cellular responses have been observed after certain flavivirus antigens have been encoded in the host by recombinant DNA technology. For example, Parrish et al. (1991) created a panel of recombinant vaccinia viruses (VVs), which together express cDNA of the entire KUN genome.

Reconstitution experiments of immune spleen cells from flavivirus-infected mice have shown that T cells play a protective role under the following conditions: 1) When there is adoptive transfer of splenocytes up to 2 weeks after immunization. If JEV escapes immune surveillance during this period, a persistent infection may result (Mathur et al., 1982). 2) When immune T cells are given before flaviviral neuroinvasion has occurred (Monath, 1996). 3) When there is a transfer of more than 10^8 cells. Such a transfer is needed locally to prevent encephalitis during JEV inoculation into the brain of mice (Mathur et al., 1982). In several cases, the peripheral transfer of immune T cells to infected hosts does not reduce mortality after flavivirus infection, which suggests that cell-mediated immunity is of lesser importance during the recovery from neurotropic

arbovirus infections than when there is peripheral transfer of antibodies (Camenga et al., 1974).

In vivo studies of the T-cell response against flaviviruses have been done by the use of serum against T cells and by the use of nude (nu/nu) athymic mice. Hall et al. (1996) found that depletion of $CD8^+$ T cells in mice immunized with vaccinia expressing MVE NS1 abrogated protection against MVE challenge, but not in mice immunized with vaccinia expressing PrM-E. Lad et al. (1993) showed that JEV infection in nu/nu mice, athymic littermates (+/nu) and Swiss syngeneic mice only produced sickness in nu/nu mice. This suggests that functional T cells play a role in the induction of active immunity as well as in the protection against JEV infection in mice (Lad et al., 1993). However, T-cell help is required to switch B-cell immunoglobulin synthesis to the IgG isotype (which has a virus-neutralizing property) (Uren, 1987; Mathews et al., 1991; Mathews et al., 1992). Thus, the lack of protection of nu/nu mice from JEV challenge could be due to the lack of T-cell help for the proper Ab response. Selective depletion of T lymphocytes with antiserum can potentially cause flaviviral encephalitis, as well as prolong survival time (Monath, 1986). However, the protective effect of immune spleen cells in JEV-infected mice can be abolished by pretreatment of the immune T cells with anti-Thy 1.2 antiserum and complement (Mathur et al., 1982). There are also cases where immunosuppression results in protection. For example, various research projects have shown that when mice are immunosuppressed during infection with encephalitic flaviviruses, they will have a prolonged survival and a reduced mortality compared with the immunocompetent mice, and they will show less inflammatory cell infiltration into the CNS (Hirsch and Murphy, 1968; Camenga and Nathanson, 1975; Semenov, 1975; Semenov et al., 1975). Thus, the role of the immune response in the protection or contribution to flavivirus pathogenesis is still unclear.

The $CD8^+$ T-cell response

$CD8^+$ T cells that are MHC class I – restricted are the most important cytotoxic T (Tc) cells. They exert their effector function by two very different mechanisms, one mediated by cytokines, and the other mediated by cytotoxic molecules that program their targets to undergo apoptosis (Rouvier et al., 1993; Kagi et al., 1994b; Lowin et al., 1994). The nature of the pathogen–host interaction may determine which $CD8^+$ T effector mechanisms are required for immunity (Harty et al., 2000).

Generation of Tc cells in the spleen has been demonstrated in mice that were peripherally infected with live encephalitogenic flaviviruses such as WNV, KUN, MVE and JEV (Gajdosova et al., 1981; Kesson et al., 1987; Parrish et al., 1991; Lobigs et al., 1994; Lobigs, 1997; Regner, 2001). The WNV genes that code the antigenic peptide determinants recognized by Tc cells have been identified using VV-KUN recombinants in a similar way as they were identified for $CD4^+$ T cells (Parrish et al., 1991; Hill, 1993). The dominant determinant in H-2^k, H-2^d, H-2^q and H-2^s haplotypes has been mapped to the NS3/NS4A region, the source of the K^k-restricted peptide determinant of the Tc-cell response against MVE has been mapped to the NS3 protein (Lobigs et al., 1994), and the sources of the MVE-specific D^k- and D^d-restricted responses have been mapped to the NS4B and NS2B-3 proteins, respectively (Lobigs, 1997). Takada (2000) investigated the Tc cell response to JEV, and also found that determinants that are derived from different viral gene products are recognized by flavivirus-immune Tc cells (depending on the haplotype of mice used). They concluded that an effective vaccine should encompass the whole virus genome in order to protect the human outbreed population.

The induction of memory Tc cells to flavivirus of the JEV serocomplex in mice does not last for their entire life (Mullbacher and Flynn, 1996). *In vitro* stimulation of *in*

vivo-primed splenocytes that will generate secondary virus-immune Tc cells only occurs efficiently if it is done approximately 2 weeks after priming (Kesson et al., 1988; Lobigs et al., 1996). This may mean that successful *in vitro* boosting only occurs when Tc cells are still in an activated state, and that they become refractory at a later stage (after encountering flavivirus-infected cells). This unresponsiveness to viral restimulation of memory flavivirus-immune Tc cells may be due to the increased MHC class I expression on flavivirus-infected stimulator cells, which results in an increased presentation of viral and self peptides (Lobigs et al., 1996). Increased expression of self peptides in this situation may be above the threshold required for activation of self-reactive Tc cells (Blanden et al., 1987) and may lead to transient T-cell autoimmunity, followed by down-regulation of autoimmunity and Tc-cell memory responses. This is consistent with the observation that Tc cells induce high lysis of mock-infected target cells when there is restimulation of flavivirus-primed splenocytes after a lengthy period of priming (Kesson et al., 1988; Lobigs et al., 1996). Persistent memory flavivirus-immune Tc cells are generated after a primary flavivirus immunization with a recombinant subunit vaccine (Regner, 2001; Regner et al., 2001), although the protective value of vaccination by induction of Tc-cell immunity against the encephalitic flaviviruses is relatively poor due to the highly self-reactive memory Tc cells.

The $CD4^+$ T-cell response

$CD4^+$ T cells become activated and proliferate after they recognize appropriate MHC class II/peptide complexes; they mature into either Th1 or Th2, depending on the stimulatory conditions. During flaviviral infections, $CD4^+$ T cells are considered to be important to the host defense mechanism. They provide help to mount a protective Ab response and they are also required to switch B-cell immunoglobulin synthesis to the IgG isotype, which has a virus-neutralization property. (Uren, 1987; Mathews et al., 1991; Mathews et al., 1992). Cytotoxic T cells are mostly MHC class I – restricted $CD8^+$ T cells, but MHC class II – restricted $CD4^+$ T cells can also exert cytotoxicity via a direct killing process through the Fas pathway ($CD4^+$ Th1) or the granule exocytosis pathway ($CD4^+$ Th2) (Henkart, 1994). An exceptionally dominant $CD4^+$ T-cell cytotoxicity has been observed during flaviviral infections with dengue in humans (Kurane et al., 1989) and with JEV (Aihara et al., 1998). Expression of MHC class II antigens is also induced after *in vitro* infection of astrocytes with WNV (Liu et al., 1989b). The process that induces the unusual cytotoxic $CD4^+$ T clones during those flaviviral infections is not clear. The induction may be due to the *in vitro* restimulation or to infections with inactivated virus preparations (except virus inactivation produced by gamma irradiation which induces the classical cytotoxic $CD8^+$ T cells) (Braciale and Yap, 1978; Aihara et al., 1998). $CD4^+$ T cells do not appear to play a significant role in the CNS inflammatory response. Experiments by Liu show that leukocyte infiltration in the CNS of mice after WNV infection consist of $CD8^+$ T and NK cells but not of $CD4^+$ T cells, and that it is unclear if flavivirus-immune $CD4^+$ T cells are required for the induction and/or activation of $CD8^+$ T cells (Liu et al., 1989a).

Several studies have been done to identify the flavivirus peptide determinants that are recognized in association with MHC class II molecules by flavivirus-immune

$CD4^+$ T cells. $CD4^+$ T cells respond to peptides that are predominantly encoded within the E and the NS1 protein (Mathews et al., 1991; Mathews et al., 1992). The fact that both E and NS1 proteins are also responsible for most of the Ab responses against flaviviruses coincides with the other important function of B cells, i.e. as APCs for $CD4^+$ T cells. VV-KUN recombinants that encode the structural polyprotein region and the non-structural proteins NS1 and NS2 produce substantial responses in the $H\text{-}2^b$, $H\text{-}2^d$ and $H\text{-}2^k$ murine haplotypes (Hill, 1993). Peptide determinants encoded within the NS4B-NS5 region are also immunogenic to the same haplotypes. Murine responses against WNV, KUN and MVE show extensive cross-reactivity in $CD4^+$ MHC class II – restricted T-cell clones, and have a strong cross-reactivity after heterologous virus priming (Doherty, 1987; Uren, 1987; Mathews et al., 1991). A similar situation occurs in humans during responses against dengue infection, when serotype cross-reactive memory T lymphocytes, induced by the primary dengue virus infection, proliferate most rapidly during the second exposure to dengue virus and induce an Ab response primarily directed against the DEN type previously encountered (Rothman and Ennis, 1999). There are two mechanisms proposed to explain the detrimental role of the preexisting antiviral immunity: 1) ADE (Hawkes, 1964; Halstead, 1982), and 2) the 'original antigenic sin' (Francis, 1953; Fazekas de St and Webster, 1966a, b). These are the mechanisms by which preexisting anti-dengue immunity can be detrimental in subsequent infection with dengue virus serotypes. Infection-enhancing immunity of encephalitic flaviviruses was shown for the first time in mice that were challenged with JEV, after vaccination with killed MVE (Lobigs et al., 2003a). Thus, immunopotentiation of disease against the most important encephalitic flaviviruses (such as JEV and WNV) in humans could occur, especially in areas where other viruses of the JEV serocomplex occur as well.

1.7 Cytotoxic mechanisms

Although cytotoxic mechanisms are part of the cellular immune response, I will discuss them in this separate section because they are the specific study objects of this thesis. Cytotoxic mechanisms are of primary importance during infection by viruses, due to the capacity of cytotoxic T cells to detect intracellular pathogens. The cells that are mainly responsible for cytotoxicity are the $CD8^+$ T cells and NK cells, which express both mechanisms of cytotoxicity. $CD4^+$ T cells are also capable of lysis, but to a lesser extent than the NK cells: the Th1 cells express FasL and the Th2 cells elicit the granule exocytosis pathway (Henkart, 1994; Kagi et al., 1994b). The recognition of an APC via the TCR induces the naïve T cell to begin the synthesis of cytolytic proteins and the specialized compartments in which these proteins are stored (Griffiths and Argon, 1995).

Two mechanisms of lymphocyte-mediated cytotoxicity have been demonstrated at the molecular level: 1) the granule exocytosis pathway, which is dependent on a pore-forming molecule (perforin) and on granule-enzymes (granzymes); and 2) the upregulation of Fas ligand (FasL or CD95L) on the killer cell, which can initiate a programmed cell death through the aggregation of Fas receptor (FasR or CD95) on target cells (Kagi et al., 1994a). The two mechanisms of cytotoxicity can be distinguished experimentally in effector cells: while the degranulation pathway is calcium-dependent, the cell death that follows the engagement of Fas is calcium-independent. Both pathways are activated in response to signals from the TCR and lead to an apoptotic death of the target cells (Fig. 1.5). In most standard cytotoxic assays, the dominant mechanism involved in the elimination of virus-infected cells is the granule exocytosis pathway. As the assay time becomes longer, the Fas pathway can become

dominant, e.g. in overnight cytotoxicity assays this pathway can be very effective (Henkart, 1997; Simon et al., 1997; Mullbacher et al., 1999a; Mullbacher et al., 2002).

The availability of mice with different combinations of mutations — perforin knockout (perf-/-) mice, granzyme A and B knockout (gzmA-/- or gzmB-/-, respectively) mice, Fas receptor mutant (*lpr*) mice and Fas ligand mutant (*gld*) mice — provides an excellent means to assess the importance of the degranulation and the Fas pathway of cytotoxicity during the infection with different intracellular pathogens. The granule exocytosis model was initially confirmed when Kagi et al. (1994b) observed that there was low cytotoxic activity in the activated lymphoid cell populations of effector cells from perf-/- mice. When they used perf-/- cells as effector cells, some cytotoxicity still occurred in wild-type cells, but not in *lpr* cells. They observed that when effectors were unable to exert perforin-based lysis and when targets were unable to be lysed through the Fas pathway, there was no other mechanism that operated (Kagi et al., 1994b). Based on the use of perforin or granzymes (gzms) knockout mice, it is evident that the granule exocytosis (or degranulation) pathway contributes to the resistance against certain virus infections (Kagi et al., 1995; Mullbacher and Flynn, 1996; Mullbacher et al., 1999b; Harty et al., 2000). Perforin-dependent cytolysis is not required for resistance against cytolytic infections such as vaccinia virus (Kagi et al., 1996), Semliki Forest virus (Kagi et al., 1995), vesicular stomatitis (Kagi et al., 1995) and cowpox virus (Mullbacher et al., 1999b) and even contributes to pathology during these infections.

Deletion of LCMV-specific $CD8^+$ T cells in perf-/- mice is less efficient for protection than it is in C57Bl/6 mice, where mortality is reduced through the depletion of $CD8^+$ T cells. These findings indicate that, on the one hand, the perforin-dependent

cytolysis is important for virus clearance and that, on the other hand, it is capable of mediating pathology (Harty et al., 2000).

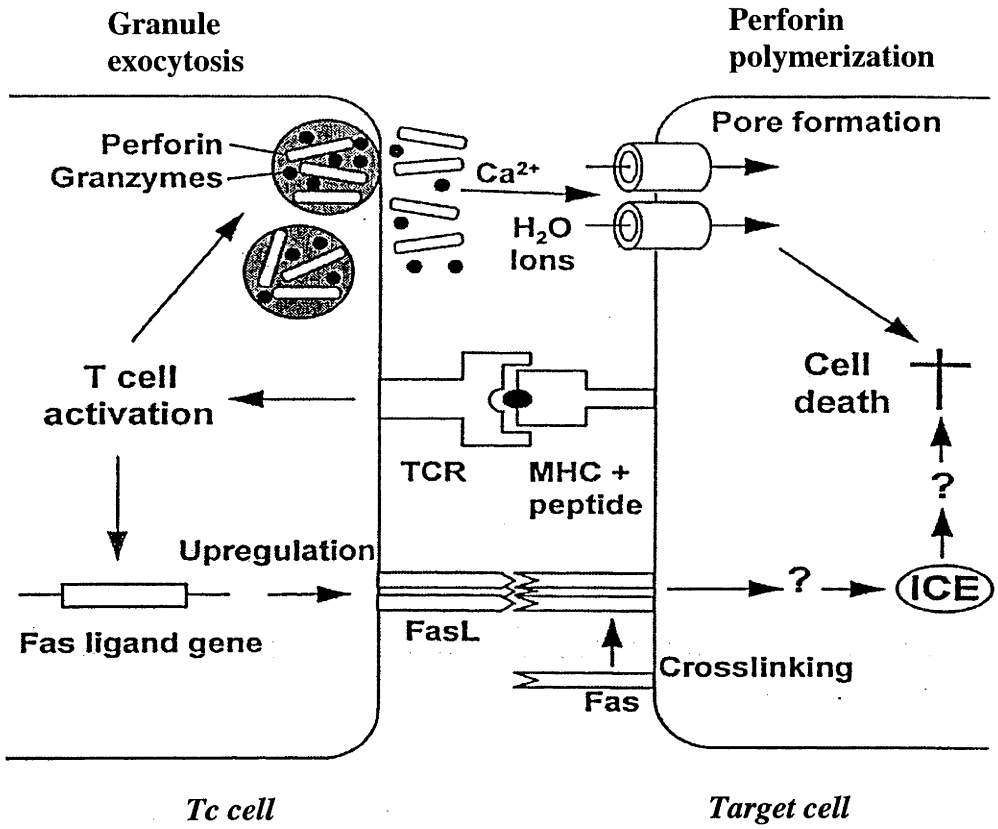

Fig. 1.5 Schematic representation of the two pathways of Tc-cell-mediated cytotoxicity. For the perforin/granzymes- and Fas-dependent pathways, the engagement of the specific TCR complex and the T activation are primary events. On the one hand, the perforin-dependent granule exocytosis pathway is initiated at the interface between effector T cell and target cell by the accumulation of cytoplasmic granules that contain perforin and granzymes. This is followed by directed exocytosis of these granules upon the target cell. Compared with the granular compartment, the extracellular space has an elevated free Ca^{2+} concentration, which induces a conformational change of the perforin molecules. This renders the perforin molecules amphipathic and enables them to insert into the target cell membrane so that it becomes permeable to water and small ions. Eventually, this permeabilization will, in combination with the effects of molecules such as granzymes that enter through the polyperforin pores into the target cell cytoplasm, lead to the death of the target cell. On the other hand, the Fas-dependent pathway is initiated by upregulation of Fas ligand expression on the T cell. Binding and cross-linking of the Fas ligand molecule, which is most probably present on the membrane in a trimeric form, with Fas molecules on the target cell leads to the induction of apoptosis. A death-inducing cytoplasmic domain on the Fas molecule triggers an apoptosis program. This program probably involves several interacting molecules, one of which may be interleukin converting enzyme (ICE) and/or a related protease. Modified from (Bustamante, 1986; Kagi et al., 1996; Chen and Cosgriff, 2000).

1.7.1 The degranulation pathway of cytotoxicity

During the degranulation pathway of cytotoxicity, perforin and granzymes from the cytotoxic lymphocytes trigger apoptosis in the target cell. Perforin molecules dissolve and undergo a calcium-triggered conformational change, which allows them to insert into the lipid membranes of the target cell. There they aggregate and form pores that can let ions and macromolecules, including granzymes, pass (Henkart, 1994). Cytotoxic lymphocytes need to inflict more than membrane damage to kill their targets *in vivo*, due to the fact that many cells can repair membrane damage (Henkart, 1994). Efficient lysis by the granule exocytosis pathway requires the coordinated delivery of perforin and granzymes into the target cell (Harty et al., 2000). Granzymes (gzms) are serine proteases, the major protein components of the lytic granules found together with perforin in Tc and NK cells. Granzymes enter the target cell and trigger an 'internal disintegration' pathway, which in turn leads to damage to the target cell (mainly caused by DNA fragmentation) (Henkart, 1994). Four gzms in humans (A, B, H and K) and seven gzms in mice (A–G) have been described. Granzymes A and B can also act directly on critical death substrates when they bypass all of the ICE-like proteases (Pham and Ley, 1997). Mice that lack gzm A and gzm B are as incapable as perf-/- mice to control primary ectromelia infection. As well as perforin, gzms A and B are indispensable for the protection against natural viral pathogens (Mullbacher et al., 1999a). Granzyme-A deficient mice have an essentially normal Tc-cell function, probably because gzm A is only involved in late cytotoxicity, which induces apoptosis approximately 14 h after Tc-target-cell contact. In Tc cells, gzm B is the most abundantly expressed gene in the cluster. Gzm B is absolutely required by Tc cells and NK cells and is used for the rapid induction of DNA fragmentation, which induces apoptosis of allogenic target cells after 15 min of incubation (Henkart, 1994). Granzyme

B activates the p34cdc2 kinase, which initiates the dissolution of the nuclear envelope and induces the cleavage of ICE family proteases (which are the effectors of apoptosis) (Henkart, 1994).

1.7.2 The Fas pathway of cytotoxicity

Fas (for fibroblast-associated) is a transmembrane receptor that belongs to the NGF/TNF receptor superfamily. In normal circumstances, the Fas system is constitutively expressed in the B, T and NK cells. Fas-receptor-mediated cell death (apoptosis) requires Fas receptor cell-surface expression at sufficient density, multimerization by a soluble or membrane-bound Fas ligand (FasL), and an apoptosis-sensitive cell in which anti-apoptotic programs have been switched off (Krammer et al., 1994). Fas-mediated apoptosis is a mechanism of immunosuppression, which down-regulates the excess in the immune response to prevent the uncontrolled expansion of specific, antigen-reactive lymphocytes. The Fas system of death signaling is mainly an immunoregulatory, rather than a destructive, function of the cells that bear the antigens of intracellular microorganisms, and is responsible for about 25% of the total cytolysis (Henkart, 1994). Lymphocytes that escape this process may replenish the pool of cells that determine immunological memory (Krammer et al., 1994). Outside the immune system, Fas is expressed in several organs such as the liver, Sertoli cells and two immunologically-privileged tissues: the eye and the testis. The Fas pathway can be subdivided into three steps: 1) reception of the death signal, 2) transduction of the death signal, and 3) activation of the cell death machinery (Pham and Ley, 1997). The engagement of the cell death surface-receptor by FasL results in apoptotic cell death, mediated by caspase activation (Bird et al., 1998; Peter and Krammer, 1998;

Tschopp et al., 1998; Waring and Mullbacher, 1999; Marti et al., 2000; Hetz et al., 2002; Sugahara et al., 2002). Caspases are widely expressed in the cytoplasm of many normal cell types as inactive proenzymes, but these proenzymes become proteolytically processed and enzymatically activated during apoptotic death. Viruses have developed various strategies to block apoptosis of infected cells, and one of these strategies is the production of caspase inhibitors. The best-studied caspase inhibitors are Crm A and baculovirus p35. Upregulation of Fas receptor after DNA damage appears to be p35-dependent. Expression of these inhibitors by transfection in numerous cell types confers protection from multiple inducers of cell death *in vitro*. *In vivo* expression of caspase inhibitors in transgenic animals also confers resistance to apoptotic death (Waring and Mullbacher, 1999).

Two mutations in the Fas pathway cause complex disorders of the immune system, and manifest themselves as lymphoproliferation and autoimmunity. One is the *lpr* (for lymphoproliferation) mutation, a recessive mutation of the Fas receptor gene, the other is the *gld* (for generalized lymphoproliferative disease) mutation of the FasL gene. Both mutations cause a very similar disease. In both cases there is unusual accumulation of T cells which express the Thy-1 antigen — an antigen that is characteristic of T cells that lack both the CD4 and CD8 surface molecules ('double negative' cells) (Falk et al., 1992). The inability of peripheral *lpr* T cells to undergo TCR-induced FasR-mediated apoptosis may cause accumulation of aberrant T cells in the mutant mice, and may result in enlarged spleen and lymph nodes. *Gld* mice that express a non-functional FasL gene may generate similar symptoms through the same pathophysiologic mechanism (Krammer et al., 1994).

To prevent the possibility of redundancy of the two major pathways of cytotoxicity, mice that lack either the perforin or the Fas subset can be generated

(Topham et al., 1997; Parra et al., 2000; Topham et al., 2001). When the Fas mechanism is absent, perforin is considered to be the main immunoregulatory mechanism. Thus, perf-/-x*gld* double-deficient mice show acute autoimmunity because they lack immunoregulation and constantly develop highly activated neutrophils. Because of the presence of $CD8^+$ T cells in the lymph nodes, spleen, pancreas, liver and kidneys of these mice, they suffer weight loss and ultimately death at an age between 4 and 16 weeks (Spielman et al., 1998; Kagi et al., 1999). About one third of the double-deficient mice die when they are between 6 and 9 weeks old (Licon Luna, 2002), while the other two thirds of perf-/-x*gld* mice can possibly be used to conduct experiments.

There are no known studies that relate the perforin-Fas mechanisms of cell-mediated cytotoxicity to the encephalitic flaviviruses. However, the rare $CD4^+$ T-cell cytolytic function seems to be responsible for some of the pathology that occurs during dengue hemorrhagic fever (DHF) in humans (Kurane et al., 1989). $CD4^+$ cytotoxic T clones may contribute to the immunopathology observed after secondary dengue virus infections through direct cytolysis and/or cytokine production (Gagnon et al., 1999). Clones from dengue virus capsid-protein specific $CD4^+$-cytotoxic T lymphocytes are capable of mediating lysis of cognate, antigen-presenting target cells through a mechanism that primarily involves perforin, while lysis occurs through the Fas mechanism of cytotoxicity. $CD4^+$ T-cell clones also produce IFN-γ, TNF-α and TNF-β (Gagnon et al., 1999). Studies of flavivirus infections in mice that are deficient in different combinations of the subsets of the granule exocytosis- or the Fas-mediated pathway of cytotoxicity will enable the *in vivo* examination of their respective contributions to virus control and pathogenesis of encephalitis.

Chapter 2

Materials and Methods

2.1 Mice

The Animal Breeding Facility of The John Curtin School of Medical Research (JCSMR) at The Australian National University (ANU) in Canberra supplied C57Bl/6 (H-2^b) mice. Wild-type and mutant mice were obtained from the JCSMR Animal Breeding Facility and from external sources (Table 2.1). Animals were housed under controlled specific pathogen free (SPF) conditions that included testing of sentinels for a standard panel of pathogens. All experiments were conducted in accordance with the guidelines of the ANU Animal Ethics Committee.

2.1.1 Mutant strains

Beige mice

Beige mice are mutant C57Bl/6 mice that lack natural killer (NK) cell activity (NK cells are lymphocytes that are non-T and non-B). Beige mice have normal numbers of NK cells, but these cells are not active (Roder and Duwe, 1979). On the other hand, the activity and the number of NK T cells in beige mice is normal (Bannai et al., 2000). Beige mice were purchased from the Animal Resources Centre (ARC), Western Australia.

Table 2.1 Mutant mouse strains (C57Bl/6 background, H-2^b haplotype) used for MVE-pathogenesis studies

Mouse strain	Mutation	Immunodeficiency in	Reference
[a]beige	Spontaneous mutation in the *bg/bg* gene	NK-cell cytotoxicity	(Roder and Duwe, 1979)
[a]RAG-1-/-	Recombinase-activating gene knockout	B- and T-cell maturation	(Mombaerts et al., 1992)
[b]β_2-M-/-	β_2-microglobulin knockout	CD8$^+$ T cells	(Koller et al., 1990)
[b]IFN-γ-R-/-	Gamma interferon-receptor knockout	IFN-γ-receptor	(Huang et al., 1993)
[b]perf-/-	Perforin gene knockout	Degranulation pathway of cytotoxicity	(Kagi et al., 1994)
[b]gzmAxB-/-	Granzyme A and B gene knockout	Degranulation pathway of cytotoxicity	(Simon et al., 1997)
[b]perfxgzmAxB-/-	Combination of above two	Degranulation pathway of cytotoxicity	(Mullbacher et al., 1999a)
[b]*gld*	Fas ligand point mutation	Fas pathway of cytotoxicity	(Roths et al., 1984)
[b]*lpr*	Fas receptor point mutation	Fas pathway of cytotoxicity	(Samelson et al., 1986)
[b]perf-/-x*gld*	Perforin and Fas ligand mutations	Degranulation and Fas pathways of cytotoxicity	(Spielman et al., 1998)

[a] ARCWA: Mice purchased from the Animal Resources Centre, Western Australia.

[b] JCSMR: Mice bred and kept under SPF conditions in the Animal Breeding Facility of The John Curtin School of Medical Research.

Recombinase-activating gene-1 knockout mice

The recombination activating genes (RAG) are an essential component of the site-specific V(D)J recombinases (Mombaerts et al., 1992) that are required to produce immunoglobulins and TCR molecules. No mature B or T cells are produced in RAG-1-/- mice, but NK and macrophage cell numbers and function remain normal (Wu and Perlman, 1999). RAG-1-/- mice were purchased from the Animal Resources Centre (ARC) in Western Australia, and maintained under standard conditions (including autoclaved food, water, bedding and micro-isolator cages).

ß$_2$-microglobulin knockout mice

Mice that are homozygous for a targeted disruption of the ß$_2$-microglobulin (ß$_2$-M-/-) gene, which is required for MHC class I antigen presentation (Koller et al., 1990), were bred and maintained in the animal facilities of the JCSMR.

Interferon-gamma-receptor knockout mice

Mice that lack the IFN-gamma receptor (IFN-γ-R-/-) have no overt anomalies, and their immune system appears to develop normally, however, IFN-gamma is necessary for a normal antigen-specific response (Huang et al., 1993) and plays an important role in protection after viral infection in many cases.

Perforin knockout mice

Transgenic perforin-deficient (perf-/-) mice (Kagi et al., 1994) lack the major cytolytic molecule of the T-cell-mediated cytotoxic mechanisms. They were originally obtained from Dr M. Simon (Max Plank Institute, Freiburg, Germany) and used to establish a breeding colony at the animal facilities of the JCSMR.

Granzymes A and B knockout mice

Mice with a deficiency in both granzymes A and B (gzmAxB-/-) were generated by crossing gzmA-/- mice (Ebnet et al., 1995) with gzmB-/- mice (Heusel et al., 1994), and by subsequently intercrossing the heterozygous F1 animals. Granzymes A and B have been implicated in Fas-independent nucleolytic and cytolytic processes that are exerted by cytotoxic T (Tc) cells (Simon et al., 1997).

Perforin, granzymes A and B knockout mice

The triple knockout perfxgzmAxB-/- mice are a result of the backcross of perf-/- mice with gzmAxB-/- mice and the subsequent intercrossing of the heterozygous F1 animals (Mullbacher et al., 1999b). These mice were maintained at the JCSMR under SPF conditions.

Fas ligand mutant mice

Mice that bear an autosomal recessive mutation in the Fas ligand locus (*gld*) express a generalized lymphoproliferative disease that becomes apparent at

approximately 12 weeks of age (Roths et al., 1984). *Gld* mice were originally obtained from the Centenary Institute, Sydney, Australia.

Fas-receptor mutant mice

Fas-receptor deficient mice that are homozygous for the *lpr* (lymphoproliferation) mutant gene develop a disease that is characterized by massive lymphadenopathy. With increasing age, the lymphoid organs in these mice are replaced with a greatly expanded population of abnormal T lymphocytes (double negative $CD4^-CD8^-B220^+$) (Samelson et al., 1986). These mice also suffer from autoimmune disease such as systemic lupus erythematosus by producing autoantibodies (Lake and Staines, 1988; Rathmell and Goodnow, 1994). *Lpr* mice were initially obtained from The Walter and Eliza Hall Institute (WEHI), Melbourne. I used both *lpr* and *gld* mice, most at an age of six weeks, when lymphoproliferative disease was minimal.

Perforin and Fas ligand mutant mice

Mice with a deficiency in perforin and Fas ligand (perf-/-x*gld*) cannot be established as a strain because females are infertile due to tissue destruction by monocyte, macrophage and $CD8^+$ T cell infiltration in the uterus (Spielman et al., 1998). Moreover, females and males die between four and 12 weeks of age, due to severe mononuclear infiltration in the pancreas, liver, kidneys and spleen (Kagi et al., 1999). Thus, in order to obtain the double-deficient mice, breeders that are heterozygous for perforin (perf+/-) and homozygous for the Fas ligand (*gld*) mutation were crossed to generate the 25% of progeny that are homozygous for both perforin-/- and *gld* genes.

Tail biopsies were taken for DNA genotyping by PCR. The mice that were homozygous for the perforin deficiency (perf-/-) were kept for experimentation.

2.1.2 Tail biopsies and DNA extraction

The personnel from the animal facilities provided tail biopsies (3–5 mm) from weanling offspring of perf+/-x*gld* mice. I added the tissues to Eppendorf tubes that contained 500 µl of digestion buffer (100 mM Tris-HCl, pH 8.0, 5 mM EDTA, 0.1% SDS, and 3.5 µl of 20 mg/ml proteinase K) (Boehringer-Mannheim, Indianapolis, IN). The digestion was performed in a rotating oven at 56°C overnight. I centrifuged samples at 8,000X g for 15 min at room temperature to pellet undigested material. After I collected the supernatant and added an equal volume of isopropanol, I centrifuged it at 10,000X g for 15 min at room temperature to pellet genomic DNA. I washed the pellet once with 70% ethanol, and I dried the DNA samples in a Savant SpeedVac concentrator (model SVC-100H) for approximately 10 min. The DNA pellet was then resuspended in 200 µl of TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA).

2.1.3 Genetic analysis by Polymerase Chain Reaction (PCR) to identify mice that are homozygous for the perforin mutation

Genomic DNA was subjected to amplification by PCR and analyzed for the perforin mutation, as described in Ebnet et al, (1995).

Perf-/-x*gld* mice had to be obtained by crossing mice that are homozygous for the *gld* gene and and mice that are heterozygous for the perforin gene, to generate mice that are homozygous for both nonfunctional genes. Because the breeders I used were

confirmed to be homozygous for the *gld* point mutation, I did not need to do further DNA testing for that mutation in the offspring.

For detection of the perforin mutations in the double-deficient mice, I amplified DNA by PCR, using the following primers: JT98 (sense direction) 5'-CCA CTC CAC CTT GAC TTC AAA AAG GCG-3, JT272 (antisense direction) 5'-TGG GCA GCA GTC CTG GTT GGT GAC CTT-3'; and 01neo2A (antisense direction) 5'-CGG AGA ACC TGC GTG CAA TC-3'.

PCR amplifications were performed in reactions that contained 0.2 μM of the JT98 primer, 0.4 μM of JT272 primer and 0.2 μM of the 01Neo2A primer. I incubated a 1 μl sample of genomic DNA with the primers and 2.2 units (U) of Taq DNA polymerase (Biotech) in a mixture of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, and 50 μM of each deoxynucleoside triphosphate. The reaction mixtures of 25 μl were overlaid with 20 μl of mineral oil (Sigma). I carried out the PCR amplifications in an automated thermal cycler (Perkin-Elmer-Cetus, Emeryville, CA) by using one denaturation cycle at 94°C for 5 min, five amplification cycles at 94°C for 30 sec, 56°C for 30 sec and 72°C for 2 min 30 sec, followed by 28 cycles at 94°C for 30 sec, 56°C for 30 sec and 72°C for two minutes, one extension cycle at 72°C for five minutes, and then cooled the mixtures at 4°C.

PCR products were analyzed by electrophoresis in 2% agarose gels (Sigma). The sizes of the products obtained were 660 bp for the perf+/+ mice (product obtained by flanking primers JT272 and JT98) and 975 bp for the perf-/- mice (product obtained by flanking primers JT272 and 01Neo2A) (Fig. 2.1). Genomic DNA from mice that are heterozygous for the perforin gene (perf+/-) was amplified as double bands of the sizes above. I used a lambda DNA/*Eco*RI+*Hin*d III size marker (Promega).

2.2 Tissue culture

2.2.1 Tissue culture for virus titration

The cell line I used for virus titration was derived from African green monkey kidney (Vero). Vero cell cultures were maintained in Eagle's minimal essential medium plus non-essential amino acids (EMEM; Gibco), 5% heat-inactivated fetal calf serum (FCS; CSL limited, Parkfield, Australia), 1 mM L-glutamine (Gibco) and antibiotics (PSN: 30 μg/ml penicillin G, 50 μg/ml streptomycin sulphate and 50 μg/ml neomycin sulphate, all from Sigma). Cells were kept at 37°C in a humidified atmosphere of 5% CO_2 in air.

2.2.2 Tissue culture for virus propagation

C6/36 cells of *Aedes albopictus* mosquito (Igarashi, 1978) were cultured in 60 x 15 mm tissue-culture dishes (Falcon). The cells were incubated in a humidified atmosphere of 5% CO_2 at 28°C in EMEM medium, supplemented with 7% heat-inactivated FCS for growth and maintenance.

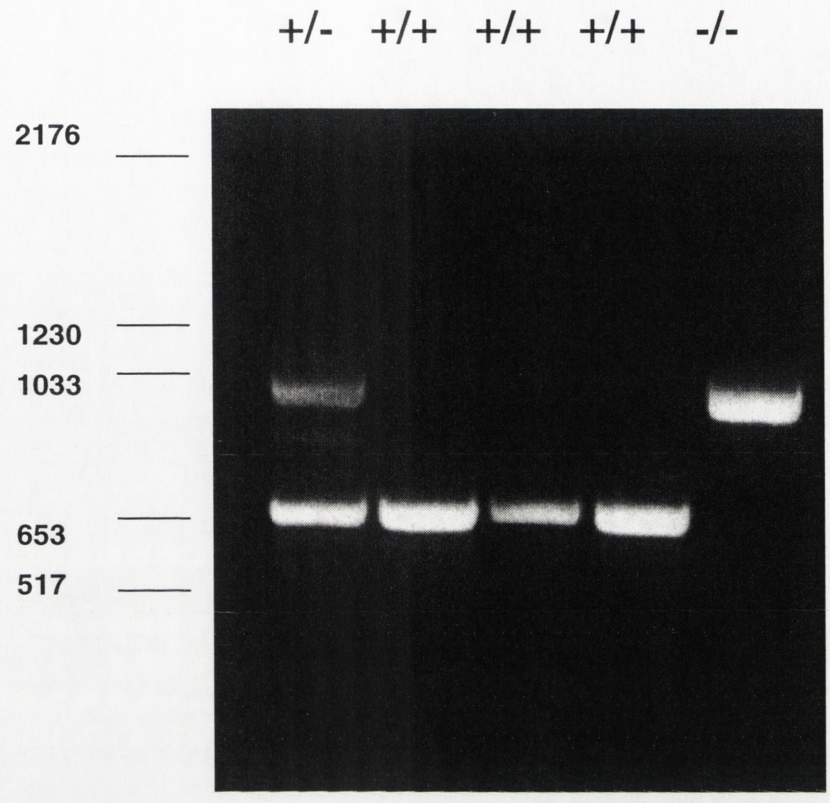

Fig. 2.1 PCR screening for the perforin mutation. DNA for PCR was prepared from tail biopsies of the offspring from backcrossed perforin heterozygous – *gld* homozygous mice, as described in Ebnet et al. (1995). The PCR product from the wild-type allele is 660 base pairs (bp) and the PCR product from the perforin mutation is 975 bp. The positions of ladder DNA size standards are shown on the left and are measured in bp. One heterozygous (+/-), shown as a double band, three wt (+/+), and one homozygous for the perforin knock out (-/-) are shown.

2.2.3 Tissue culture for assays of cytotoxicity

Mouse B10A.2R cells (fibrosarcoma; H-2K^kD^b) (abbreviated as 2R) and B10A.5R cells (fibrosarcoma; H-2K^bD^d) (abbreviated as 5R) were maintained in EMEM, supplemented with 5% heat inactivated FCS at 37°C in a humidified atmosphere of 5% CO_2 in air, and used as targets during cytotoxic assays. I infected cells with virus or left them uninfected, and then labeled them with ^{51}Cr as described in Hill et al. (1992) and Kesson et al. (1988).

2.3 Viruses used and methods of preparation

2.3.1 Viruses

Working stocks of MVE (prototype strain MVE-1-51; French (1952) were prepared by either infecting suckling mice intracerebrally (ic) or by infecting a C6/36 cell culture. The MVE stock I used had previously been passed 15 times through suckling mouse brains, which did not reduce virulence in mice infections (Lobigs et al., 1986).

Vaccinia (VV) recombinant viruses (VV-STR, VV-prM/E, VV-E, VV-NS, VV-NS1-NS3 and VV-TK$^-$) have been described previously in Lobigs et al. (1994), and I used these viruses accordingly. Dr Mario Lobigs provided all the recombinant viruses.

2.3.2 Preparation of virus stocks from mouse brains

One- to two-day-old (newborn) Swiss mice were infected ic with approximately 3 µl of virus (10^5 PFU/ml). Brains were collected when the mice started to show signs of disease (lack of weight gain, slow motion) at 2–3 days post-infection (pi). I added

nine volumes of virus diluent (VD, consisting of Hanks' balanced salt solution [HBSS], supplemented with 0.2% of bovine serum albumin [BSA] and 20 mM of Hepes buffer, pH 8.0) and homogenized the brains with a 15-ml glass homogenizer and a tight fitting plunger. Suckling mouse brain homogenates were clarified by centrifuging for five minutes at 4°C at 18,000X g on a high-speed bench-top centrifuge (Heraeus Biofuge 28 SR).

2.3.3 Preparation of virus stocks from mosquito cell culture

Tissue-culture-grown virus stock was prepared by infecting monolayers of C6/36 cells at a multiplicity of infection (MOI) of 0.5. Medium was replaced at day 3 pi to avoid nutrient exhaustion. Once approximately 70% of the cells showed cytopathic effect (CPE), the supernatant was harvested, and 400 µl of 1 M Hepes (pH 8.0) was added to every 20 ml of supernatant. To separate the cells and debris from the virus-containing fluid, the mixture was centrifuged (10,000X g) at 4°C. Virus stocks were frozen in 0.5 ml aliquots at -70°C until they were used (virus stocks were thawed once only). Titration was done by plaque assay in Vero cells as described in section 2.6.

2.4 Parenteral injections

Virus aliquots were removed from -70°C as required, thawed rapidly in a 37°C water-bath and diluted in VD. I carried out the inoculations with specified virus dilutions that were kept in an ice bath.

2.4.1 Intravenous injections

Mice were inoculated with 0.1 ml of virus intravenously (iv) by the tail vein route, using a 26-G needle and a 1-ml tuberculin syringe.

2.4.2 Intraperitoneal injections

For cytotoxic assays or antibody, respectively and *in vivo* NK-cell depletion studies, I inoculated mice intraperitoneally (ip) with virus using a 26-G needle and a 1-ml tuberculin syringe.

2.4.3 Intracranial injections

Intracranial (ic) inoculations with a 30-G needle and a 1-ml tuberculin syringe were carried out under light ether (Sigma) anesthesia in adult mice for mortality studies and in newborn mice for preparation of virus stocks. For mortality studies, mice received a volume of 30 µl ic.

2.5 Tissue collection

At indicated times after inoculation, I anesthetized mice with ether and killed them by cervical dislocation. With the mice lying on their dorsal surface, I opened the thorax and collected the blood from the heart. I transferred the blood to an Eppendorf tube to separate the serum from the blood cells by centrifugation (18,000X g for five minutes at 4°C), and stored it at -70°C. Next, I opened the peritoneal cavity and

aseptically collected spleen, kidneys, lymph nodes (mesenteric and inguinal) and liver. I then dissected muscle of the hind limb (*rectus femoris*) and, finally, removed the brain. I divided tissues in three aliquots: one for plaque assay, another for RNA extraction and a third, fixed in 10% neutral buffered formalin, for histology studies. The tissues that were collected for plaque assay and RNA extraction were snap frozen in dry ice or liquid nitrogen and stored at -70°C. I obtained blood samples from live mice by paraorbital or tail bleeding and separated serum from blood cells by centrifugation at 18,000X g at room temperature for five minutes. I then stored it at -20°C until it was used for analysis in ELISA.

2.6 Determination of MVE titers in virus stocks and tissues

2.6.1 Tissue processing

Organs were weighed and homogenized for plaque assay in VD to obtain a 10% (w/v) suspension. Homogenization was achieved by passing the tissues (muscle, spleen and lymph nodes) through a metal strainer or, in the case of soft tissues (brain and liver), through gradually smaller sized needles (from 18 to 25 G). I centrifuged the homogenates (at 18,000X g for five minutes at 4°C) to remove cell debris. Aliquots were taken from the supernatants and stored at -70°C. I titrated tissues for content of virus by plaque assay in Vero cells.

2.6.2 Plaque assay for MVE-stock titration and determination of virus titers in tissues

Vero cells in 6-well plates (Linbro) were grown to 70% confluence. I removed approximately three fourths of the culture medium and infected the cells (in duplicate wells) with 0.1 ml of tissue homogenate that was serially diluted in VD on ice. Adsorption was allowed for one hour, while the samples were rocked every 15 min. I did not remove the inoculum prior to adding the overlay (4 ml/well), which consisted of 1% agar (Bacto), EMEM, 2% FCS, antibiotics (PSN as in section 2.2) and 0.25 µg/ml of fungizone (Life Technologies, Rockville, Maryland, USA). Ninety-six hours later, I added neutral red (BDH Chemicals, Poole, England) in Hanks' (0.03% w/v) for staining. The day after, I removed the stain and the overlay and counted the plaques. Virus titers were expressed as the number of plaque forming units (PFU) per gram (PFU/g) of tissue, or per milliliter (PFU/ml) of serum. The limit of detection was set at 10^3 PFU/g of wet tissue or at 10^2 PFU/ml of serum.

2.7 Reverse transcription/PCR for detection of MVE RNA

2.7.1 Preparation of total RNA from tissue samples

Total RNA was prepared from different tissues of infected mice using a procedure that was modified from Promega (Technical bulletin 087). Initially, I homogenized the frozen tissue samples in 10% w/v PBS, but this process frequently caused RNA degradation in hematopoietic organs (spleen and lymph nodes). To reduce the risk of RNA degradation, I then employed a second extraction method. For this method, I added a small portion of tissue (approx. 3 mm^3) directly to 0.5 ml of guanidine thiocyanate (Promega) for five minutes on ice, and subsequently

homogenized the tissue samples with a syringe by passing it through needles of gradually smaller size (18 to 25 G). I then added 50 µl of 2 M sodium acetate (pH 4.0) and vortexed the samples. After adding an equal volume of acid-equilibrated phenol:chloroform (5:1; Sigma), I vortexed the samples for one minute, held them at 4°C for 15 min, and then centrifuged them at 18,000X g for 20 min at 4°C. I transferred the upper aqueous phase to a tube that contained an equal volume of isopropanol, mixed it, and it held at -20°C for 20–30 min. Subsequently, I pelleted the RNA by centrifuging it at 18,000X g for 15 min at 4°C. I removed the supernatant and washed the pellet twice with 1 ml of 75% ethanol. The RNA samples were dried in a Savant SpeedVac concentrator (model SVC-100H) and were resuspended in 20 µl of nuclease-free water (Sigma), before being stored at -70°C.

2.7.2 Agarose gel electrophoresis of total RNA samples

Total RNA (1 µl) that was obtained from tissues of MVE-infected mice was mixed with 5X loading buffer (20% ficoll 400, 25 mM EDTA pH 8.0, 2.5% SDS, 0.05% bromophenol blue, 0.03% xylene cyanol) and was electrophoresed in 1% agarose gels which contained 1X TBE, 0.1% SDS and 1 µg/µl ethidium bromide (Sigma). I observed two bands, corresponding to the 26S and 18S ribosomal RNA. Degradation of RNA was indicated by a smear. Viral RNA was not visible in these samples, but the clarity of the ribosomal bands gave an indication of the quality of the preparation. I used a lambda DNA/*Eco*RI+*Hin*d III size marker (Promega).

2.7.3 Preparation of a positive control for detection of MVE RNA

The MVE RNA positive control, a T7 RNA polymerase transcript, was produced from a cDNA clone of MVE-1-51 (the prototype virus) that encompassed nucleotide 1 to 5010 of the MVE genome, with an internal deletion between nucleotides 1450 and 2050 (Hurrelbrink et al., 1999). The reverse transcription/PCR amplification of the RNA positive control was 616 bp long, while the product obtained from MVE was 717 bp long. The different sizes of the PCR products reduced the risk of a false-positive interpretation. The MVE RNA was generated using T7 RNA polymerase in a reaction that contained transcription buffer (Promega), 1 mM of rNTP, DTT (10 mM), RNase inhibitor (1 U/µl; RNA guard, Pharmacia), T7 RNA polymerase (0.5 U/µl; Promega) and nuclease-free water (Sigma), in a final volume of 20 µl. I incubated the reaction for one hour at 37°C.

For purification, I treated the sample with one U of DNase I-RNase free (Pharmacia, FPLC pure) at 37°C for 15 min. I then inactivated the DNase by incubating it at 70°C for 15 min. I extracted RNA twice with equal volumes of phenol and chloroform (1:1) that was precipitated by adding one-tenth volume of 3 M sodium acetate (pH 5.6) and 2.5 volumes of ethanol. I collected RNA pellets by centrifuging the mixture at 18,000X g, washing it once in 70% ethanol, drying it and resuspending it in 20 µl of nuclease-free water. I then loaded the undiluted samples and the dilutions of 1:5, 1:25 and 1:2 in a 1% agarose gel (Sigma). Given that a minimal amount of approximately 10 ng of RNA can be visually detected in an agarose gel, the concentration of the product can be roughly estimated. I diluted the RNA to a final concentration of 10 ng/µl (6.02×10^{23} molecules per mole resuspended at 10^5 copies/µl) and stored aliquots of 2 µl at -70°C.

2.7.4 Oligonucleotide primer preparation

The JCSMR Biomolecular Resource Facility made the oligonucleotide primers and supplied them in ammonium hydroxide. I transferred aliquots of the oligonucleotide primer (100 µl) to Eppendorf tubes and added 10 volumes of n-butan-1-ol to each tube. I vortexed the samples and then centrifuged them at room temperature at 18,000X g for two to three minutes in an Eppendorf microfuge (model 5415C). All the top (organic) phase was carefully aspirated and the aqueous bottom phase, which contained the oligonucleotide, was dried for 10 min in a Savant SpeedVac concentrator (model SVC-100H). I resuspended the pellet in 100 µl nuclease-free water.

2.7.5 RT/PCR

An RNA sample (1 µl) was transcribed by combining 2.6 U of expand-reverse transcriptase (Boehringer-Mannheim), 25 pM of random hexamer primers (Promega), 0.8 mM dNTP mix in a 1X reverse transcriptase buffer (Boehringer-Mannheim), and 10 mM DTT (Promega), in a total reaction volume of 10 µl. I incubated the reactions for 10 min at room temperature, and subsequently for 30 min at 42°C. The cDNA that was generated from the reactions was stored at -20°C.

The cDNA was then amplified with two MVE-specific oligonucleotide primers: the sense-direction 23mer primer 5' ACG AAA CAC TTT CTA GTG CAT CG (nucleotides 1547 to 1612 in the MVE genome) and the antisense-direction 23mer primer 5' GGA GGA GCA TTT AGA ACC CCT CC. These primers amplify cDNA that corresponds to the positions 1615 and 2332 in the MVE genome (Dalgarno et al., 1986), with respect to the 5' terminus in the MVE genome. The product obtained was 717 bp long. Two µl of cDNA template were added to a PCR amplification mix that

combined 0.5 µM of each primer, 0.2 mM of dNTPs, 1X reaction buffer and 1 U Taq DNA polymerase (Advance Biotechnologies) in a total reaction volume of 25 µl, using a PCR thermocycler (Perkin Elmer GeneAmp PCR system 2400). The negative control contained water instead of cDNA. Thermal cycling consisted of a denaturation cycle at 94°C for 2 min, 35 amplification cycles at 94°C for 1 min, 51°C for 1 min, and 70°C for 40 sec, followed by one cycle at 68°C for 7 min.

RT-PCR products were analyzed by agarose gel electrophoresis. For this procedure, I dissolved molecular biology grade agarose (Promega) (1% w/v) in TBE buffer with ethidium bromide (1 µg/µl) though boiling it; mixed a PCR sample of 10 µl with 5X loading buffer; and used a Bio-Rad tank for electrophoresis. I used a lambda DNA/*Eco*RI+*Hin*d III marker (Promega).

The quality of the RNA samples was assessed by RT-PCR using a pair of ß-actin mRNA specific primers (Clonotech), 5'-GTG GGC CGC TCT AGG CAC CAA-3' (sense direction) and 5'-CTC TTT GAT GTC ACG CAC GAT TTC-3' (anti-sense direction). This produced a product of 540 bp. The PCR mix and electrophoresis conditions were the same as for the above reaction. I added water to the negative control. I used a DNA size marker with a low molecular weight (DNA molecular weight marker VI; Boehringer-Mannheim). The cycling temperatures were as follows: 94°C for 2 min; 30 cycles at 94°C for 45 sec; 60°C for 45 sec; 73°C for 1 min; 72°C for seven minutes and a soak cycle at 8°C.

2.8 Detection of total MVE-specific antibody (Ab) by enzyme-linked immunoabsorbent assay (ELISA)

2.8.1 Production of ELISA antigen

The MVE antigen (Ag) was produced as described in Colombage et al. (1998). Vero cell monolayer cultures were grown in 850 cm^3 roller bottles (~10^8 cells) that were infected with MVE (moi~10) at 37°C. I over-layered cells with EMEM, which contained 5% fetal calf serum (FCS), and incubated them at 37°C while rolling the bottles continuously. At 24 hours post-infection, I replaced the medium with 50 ml of EMEM, which contained 2% FCS and 20 mM of Hepes buffer (pH 8.0). At 48 hours pi, I harvested the culture fluid, replaced the medium with the same mixture as above, and subsequently conducted a second harvest at about 60 h pi. Cell debris was removed by centrifugation at 18,000X g for 10 min at 4°C in a Sorval centrifuge. I concentrated the virus in several steps. I firstly added 40% w/v polyethylene glycol 8,000 (Sigma) in NTE buffer (0.12 M Na2Cl, 0.012 M Tris-HCl, 0.001 M Na2 EDTA; pH 8.0) to a final concentration of 8%; it was then stirred for two hours at 4°C. I then recovered virus by centrifuging the mixture at 18,000X g for 30 min at 4°C. Subsequently, I drained the pellets and dissolved them in 4 ml borate saline (0.05 M borate, 0.12 M Na2Cl; pH 9.0), to make a 50-fold concentration. I added NTE buffer to the suspension until it totaled 10 ml, layered it on 2 ml of sucrose cushion (30%) and centrifuged for 3 h in a SW41 rotor at 35,000 rpm at 4°C in a Beckman ultracentrifuge. I then drained, resuspended, aliquoted and stored the pellets at -20°C.

2.8.2 Plate coating with MVE Ag

U-bottom 96-well assay plates (Titertek) were coated with 50 µl of MVE antigen that was diluted to 1/1000 in borate saline (pH 9.0). The plates were then incubated overnight at 4°C. I flicked the virus in to bleach and washed the plates once with PBS-Tween (0.05%). The plates were then ready to be used immediately or were stored for a maximum of one month at -20°C.

2.8.3 Ab titration by ELISA

The 2.2'-azino-di[3-ethyl-benzthiazoline sulphonate] (ABTS) microwell peroxidase substrate system (Kirkegaard & Perry Laboratories, Gaithersburg, Maryland, USA) was used according to the manufacturer's instructions, with minor modifications. Samples and controls that were initially diluted to 1:100 were further serially diluted by two until 1:102,400 (10 times more) in 1% BSA-PBS-Tween (Sigma). They were then incubated at room temperature for two hours. I washed the plates four times with wash buffer (0.05% PBS-Tween) and blot-dried them. I detected mouse antibodies using affinity-purified goat anti-mouse immunoglobulin, conjugated with horseradish peroxidase (50 µl/well) that was diluted to 1:1000 in 1% BSA-PBS-Tween. The plates were incubated at room temperature for one hour and then washed (as described above). I added freshly prepared ABTS substrate solution (50 µl/well) to the plates and incubated them at room temperature until color developed (approx. 20 min). I stopped the color reaction with 1% SDS and read the color immediately, using a Molecular Dynamics microplate reader at 405 nm.

The specific activity of each test serum was established as the cut-off values of the mean optical density (OD) of eight negative control wells containing sera from

naïve mice plus 3 standard deviations. The OD values of the tested sera were considered positive if they were equal or greater than the OD cut-off values and end points were calculated as the reciprocal value of the last dilution that gave a positive OD value.

2.9 Histology

Formalin-fixed tissues were taken to the Histology Unit of the JCSMR for routine paraffin-embedding, cutting, mounting on slides and staining with Hematoxylin-Eosin. I sent slides to the Max-Plank Institute, Freiburg, Germany, to be interpreted by Mr Thomas Steele.

2.10 Passive transfer of immune B and T cells

2.10.1 Generation of anti-MVE effectors

C57Bl/6 wt donor mice (12) were infected iv with $1x10^2$ PFU of MVE. I then aseptically collected groups of four spleens at day 6 pi in 50-ml tubes, that contained 20 ml of EMEM medium at room temperature. Subsequently, I made a single-cell suspension by gently pressing the spleens through a stainless steel mesh with syringe plungers. I depleted erythrocytes by hypotonic-shock treatment (I added 4.5 ml of distilled water to cell pellets for 30–45 s) and added 0.5 ml of 10X HBSS to bring the solution back to isotonicity. I then centrifuged the splenocytes at 200X g for five minutes at room temperature, washed the pellets twice with EMEM and resuspended them in 2 ml of EMEM containing 5% FCS, supplemented with 10 mM of Hepes pH 8.

2.10.2 Nylon wool cell separation

Ten-ml nylon-column syringes were prepared by loosely packing 0.6 g of sterilized and scrubbed nylon wool (Robbins Scientific). I then affixed a three-way plastic stopcock (Beckton Dickinson) and added EMEM 5% FCS until the wool was covered. I incubated the columns at 37°C overnight. Subsequently, I took the warmed columns from the incubator and let the top portion of the medium/5% heat inactivated-FCS run off. I then added the cell suspension to the columns, allowed it to enter the nylon wool bed, and stopped it just before the fluid with cells could escape. The columns were then topped with more warm medium EMEM 5% FCS, and incubated at 37°C for two hours.

Preparation of the T-cell enriched fraction

The columns were carefully removed from the incubator. I attached an 18 G needle to the cock to control the flow of the medium through the syringe. To recover the T lymphocytes, 10–20 ml of warm medium/5% heat inactivated-FCS was allowed to slowly drip (~10 min) through the syringe that was held vertically into the tube. I recovered the effluent containing the T cells (non-adherent to the nylon-wool) from each syringe, pooled it, and centrifuged it at 200X g for five minutes at room temperature. I then resuspended the enriched cells in medium that was supplemented with 0.2% BSA at 2 x 10^8 cell/ml.

Preparation of the B-cell enriched fraction

To recover the adherent B cells, I used cold medium (10°C) without FCS (FCS enhances the adherence of the B cells to the nylon) for elution. While I added medium, I detached cells by poking the nylon with a pipette and then collected the effluent, pooled it and centrifuged it at 200X g for five minutes at room temperature. I then resuspended the cells as described above.

2.10.3 Flow cytometry

To assess the phenotype(s) of the transferred splenocytes, I stained samples of 10^6 cells with anti-mouse fluorescent antibodies (Pharmingen, San Diego, CA, USA). I labeled the cells with rat anti-mouse CD8a (Ly-2) (R-phycoerythrin [R-PE] PBS diluted 1/100) monoclonal Ab, rat anti-mouse CD4 (L3T4) (fluorescein isothiocyanate [FITC] PBS diluted 1/50) monoclonal Ab, rat anti-mouse B (CD45R/B220) [R-PE] PBS diluted 1/100) monoclonal Ab, and mouse anti-NK cells/2B4 (PE, PBS diluted 1/6) monoclonal antibodies. I added 20 µl of the labeling monoclonal Ab (diluted according to the manufacturer's instructions) to each tube of cells and incubated the tubes for 20 min at 4°C. I centrifuged the tubes at 200X g for five minutes, and subsequently washed them twice with 1 ml of PBS. I then resuspended the cells in 200 µl of PBS. I did the analysis of the surface fluorescence of cells on a FACScan flow cytometer (Beckton Dickinson, Franklin Lakes, NJ, USA).

2.10.4 Adoptive transfer of anti-MVE effectors

Nylon-wool-purified anti-MVE effector B or T cells were injected iv in six mice that received 4 x 10^7 cells in a volume of 0.2 ml, after they had received 10^2 PFU iv of

MVE three days previously. Controls were: 1) six uninfected mice that received anti-MVE T or B cells; and 2) six non-transferred mice that were infected with 10^2 PFU iv three days previously. I recorded mortality for each group.

The risk of virus carry-over in the transferred nylon-wool-purified B and T cells from infected mice was tested by plaque assay. In all cases the cells from the infected donors were found to be free of any detectable virus.

2.11 Assays of cytotoxicity

Secondary *in vitro*, MVE- and VV-immune Tc cells were generated according to the methods described in other research papers (Kesson et al., 1988; Mullbacher et al., 1991; Hill et al., 1992; Regner et al., 2001).

2.11.1 Generation of effector cells

Spleens from MVE- or VV-primed mice (ip injection with 5 x 10^6 PFU) were harvested 10 days pi. By gently pressing the spleens through stainless steel mesh with syringe plungers, I obtained single-cell suspensions of the spleens. I then washed the cells twice in EMEM, supplemented with 10 mM Hepes. Subsequently, I washed the cells twice with EMEM, supplemented with 5% FCS, 2 mM L-glutamine, 10 mM Hepes, 0.2% NaHCO3 and antibiotics (PSN).

To restimulate these cells with virus, I infected one fifth of the spleen cell suspensions for one hour with 5 PFU/cell of MVE or with 0.5 PFU/cell of VV-TK$^-$, washed these suspensions three times with EMEM/5% FCS, and cultured them with the

rest of the splenocytes for five days in EMEM, supplemented with 10% FCS and 10^{-4} 2M-mercaptoethanol (culture medium).

2.11.2 ^{51}Cr-release cytotoxicity assay

The 2R and 5R target cells (2 x 10^6) were infected with MVE at an MOI of 50 PFU/cell 24 h prior to being ^{51}Cr-labeled for one hour. Another batch of 2R and 5R cells was infected with VV at an MOI of 10 for one hour, in presence of ^{51}Cr. I washed the target cells three times with culture medium and co-cultured them with titrated numbers of effector Tc cells for six hours. I performed all assays in triplicate in 96-well plates; the SEM was never greater than 5% and medium release was never greater than 22%. I infected target cells for mapping experiments with VV recombinants encoding MVE polyprotein fragments (VV-STR, VV-PrM-E, VV-pr-M, VV-E, VV-NS, or VV-NS1-NS3) as described in Hill et al. (1992) and Lobigs et al. (1994).

2.12 Statistical analyses

The mortality ratios after virus challenge were assessed using Fisher's exact test. Data in this thesis are expressed as means ± standard error of the mean (SEM). I used the student's t-test to determine the significance of the differences between the experimental and the control groups, and I used the Mann-Whitney test to determine the significance of the differences in time to death.

Chapter 3

Comparison of reverse transcription/polymerase chain reaction (RT/PCR) and plaque assay (PA) for detection of MVE replication in mouse tissues

3.1 Summary

In this chapter, the sensitivity of virus detection by reverse transcription/polymerase chain reaction (RT/PCR) is compared with the sensitivity of virus detection by plaque assay (PA), using tissues from MVE-infected mice. The results show that the RT/PCR and PA methods are equally sensitive to detect virus in most of the tissue samples that were tested. I selected PA as the preferred method for my subsequent studies because it is a simple and quantitative method for the detection of infectious viruses.

3.2 Introduction

Flaviviruses are defined as primarily neurotropic in mice. Some researchers (Murphy et al., 1968; Hase et al., 1990; McMinn et al., 1996; Andrews et al., 1999) consider that tissues other than the CNS are refractory to flavivirus replication, while other researchers (MacDonald, 1952; Huang, 1963; Monath, 1996) have shown that virus grows in extraneural organs, albeit at low levels.

Several techniques have been developed to detect encephalitic flavivirus infections: by detection of specific Abs, or by detection of viral particles via RT/PCR, quantitative real-time PCR (TaqMan) and PA. In humans, because of the short viremic phase, flaviviruses are most commonly diagnosed by detection of IgM in serum or cerebrospinal fluid (CSF) (Pinto et al., 1988; Harrington et al., 2003; Hiatt et al., 2003); however, the assay cannot differentiate between WNV, SLE, JEV and other members of the JEV serocomplex (Lanciotti et al., 2000). The RT/PCR assay on the other hand has been used for diagnosis of JEV (Meiyu et al., 1997), MVE (McMinn et al., 2000) and

WNV (Porter et al., 1993; Huang et al., 2002; Harrington et al., 2003). The RT/PCR technique is particularly important as it can provide a rapid and specific detection.

TaqMan RT/PCR has been recently developed and is also available for specific and rapid diagnosis of viruses (Mackay et al., 2002). TaqMan RT/PCR uses fluorescent DNA probes in a 5' exonuclease assay, which has been used for testing brains of autopsied humans (Lanciotti et al., 2000), serum (Bae et al., 2003; Harrington et al., 2003; Hiatt et al., 2003) and mosquito pools (Ritchie et al., 2003). TaqMan RT/PCR offers the advantage over traditional RT/PCR of increased sensitivity, increased reproducibility and better quantitation (Morris et al., 1996; Martell et al., 1999). In general, RT/PCR assays are considered to be rapid and sensitive, although they require several steps that can increase the risk of inter-tube contamination and are difficult if quantitative results are required (Wang et al., 2000). Additionally, whilst RT/PCR assays can detect virus-specific nucleic acids, they do not provide information about the infectious capability of the virion. On the other hand, the conventional method of virus titration by PA does provide quantitative assay of infective virus (important during vaccine production and control), but it is time consuming and laborious (Wang et al., 2000; Bae et al., 2003).

The fact that encephalitic flaviviruses in peripheral organs are either undetectable or at the threshold of the detection limit could, when using standard methods of virus detection such as RT/PCR or PA, be due to two factors: 1) the limitation of the method used, and 2) the poor virus growth in extraneural organs. It was important to this project to investigate if the failure to detect virus in the peripheral tissues was due to the release of virus inactivating factors, e.g. proteases and RNAses, during tissue processing and not just due to poor virus replication in those tissues. Thus, I undertook a comparison of the two different methods of virus detection, RT/PCR for

the detection of viral RNA, and PA for the detection of infective virions, and tested them on aliquots of the same tissue samples obtained from infected adult mice. I evaluated advantages and disadvantages of the RT/PCR and PA approaches in order to select the best method to investigate the kinetics of MVE replication in mouse tissues.

3.3 Results

3.3.1 Comparison of the RT/PCR and PA methods for virus detection

To determine which technique was more suitable for virus detection in particular types of tissues, the RT/PCR method was compared to the PA method. I obtained samples of brain, spleen, lymph nodes and muscle from MVE-infected mice that showed either low virus titers by PA or faint DNA bands by PCR and tested the same samples using both techniques. Six samples from each organ are shown in Figs 3.1 to 3.4. I used aliquots of each tissue sample to: 1) prepare RNA for RT/PCR, and 2) produce a 10% w/v homogenate, which is required for serial dilutions during plaque assay.

Initially, I took aliquots from homogenized tissues in PBS and used these for RNA extraction and for plaque assay. However, I observed that tissue homogenization resulted in significant RNA degradation, particularly in lymph nodes and spleen. Therefore, I reduced the extent of RNA degradation by adding approximately 3 mm^3 of frozen tissue (kept on dry ice) directly into the ice-cold guanadinium thiocyanate (the first step for RNA extraction). After an incubation period of 15 min on ice, the tissue was homogenized by passing it through gradually smaller needles (18 to 25 G). I homogenized the tissues for PA in ice-cold HBSS-BSA (pH 8.0) to reduce the loss of virus infectivity. The quality of RNA was not compromised to an extent that prevented

amplification, since the ß-actin control PCR was positive for all the samples and the same samples that were positive by PA were also positive by RT/PCR (Figs 3.1 to 3.4 B). This indicates that these primers were of adequate quality for effective RT/PCR.

RT/PCR was performed on total RNA extracts of tissue samples using a pair of E protein gene-specific primers. The cDNA product from RT/PCR was 717 bp in size. In assays performed on a positive control-RNA (cloned MVE cDNA and T7 polymerase-mediated transcription that gives rise to a 616 bp cDNA product due to nucleotide deletion), the threshold for RT/PCR amplification under the conditions used was 10^2 copies of target-RNA.

For the infectivity-based approach, I assayed the different dilutions of tissue homogenates for plaque formation, as described in section 2.6. I then compared virus titers from each tissue with the intensity of the PCR bands.

The comparison of the results of the RT/PCR and PA methods for brain tissue showed that the three samples that were positive using the PA method had MVE titers that were marginally above the detection limit of 10^3 PFU/g, while the equivalent samples, investigated with the RT/PCR method, showed clearly positive results (Fig. 3.1). Additionally, there was a direct correlation between the values of the virus titers and the intensity of the bands in the PCR results of the brain samples. The sample that had the highest titer by plaque assay (1.5×10^4 PFU) also produced the greatest yield of cDNA in RT/PCR (Fig. 3.1, sample 3).

The comparison of the results of the RT/PCR and PA methods for infected spleen is shown in Fig. 3.2. I obtained positive results by both methods for samples 1, 2, 3 and 5, despite some degradation to the RNA extracts (smeary appearance in agarose gel electrophoresis; data not shown).

The results of RT/PCR amplification and virus titration by plaque assay for lymph node samples are shown in Fig. 3.3. I obtained positive results for samples 1 and 3 by RT/PCR but not by PA, although the cDNA products of PCR bands were barely detectable. There was a direct correlation between the results of RT/PCR and PA for samples 2 and 4 (negative) and sample 5 (positive), the latter showing a titer of 10^3 PFU/g and a clear band in the RT/PCR result.

In general, I obtained few MVE-positive muscle samples by RT/PCR or plaque assay. Six samples are shown in Fig. 3.4. Only sample 3 produced a clearly positive result by both RT/PCR and by PA with a titer of 1.3×10^3 PFU.

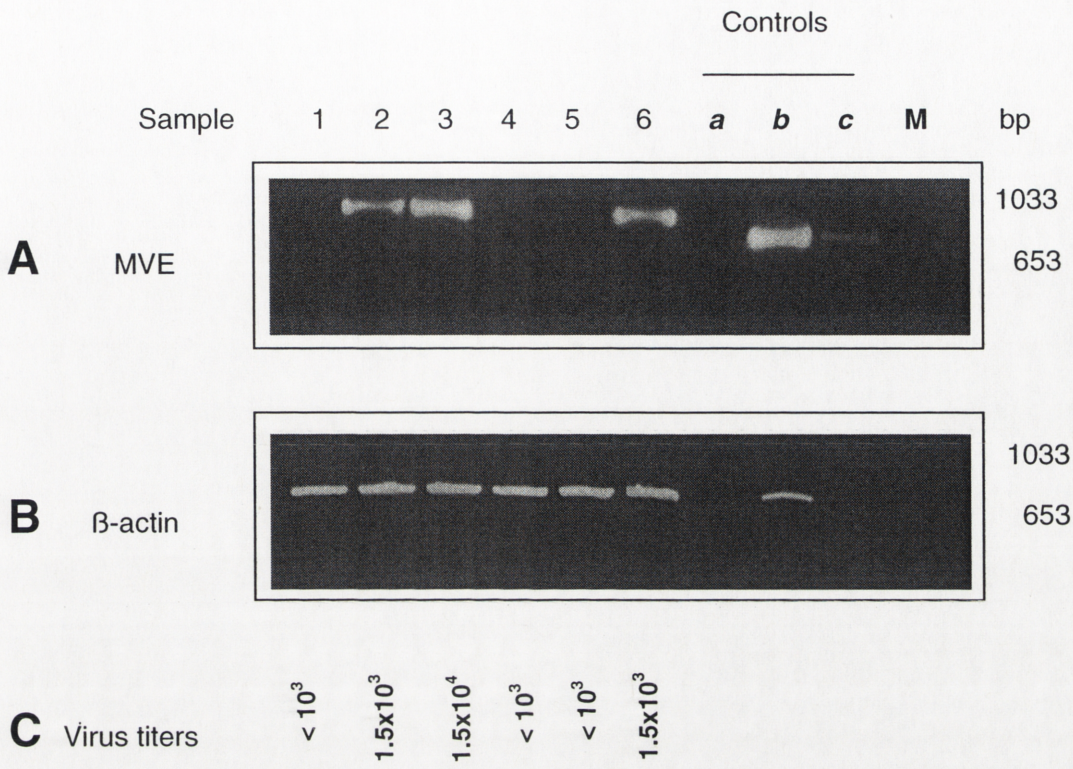

Fig. 3.1 A comparison of the results of RT/PCR and PA of brain samples taken from mice that were infected iv with 10^2 PFU of MVE

Brain samples (1–6), controls (***a–c***) and DNA molecular weight marker (**M**).

A, MVE-specific RT/PCR. Controls: ***a,*** reagents control (water); ***b,*** PCR amplification of 10^5 RNA copies; ***c,*** PCR amplification of 10^2 RNA copies. Products of 717 bp from infected tissues and 616 bp from the positive control were amplified by MVE-specific RT/PCR.

B, ß-actin specific RT/PCR (540 bp). Controls: ***a,*** reagents control (water); ***b,*** positive control; ***c,*** blank.

C, virus titers obtained from infection of Vero cell monolayers, in PFU/g of wet tissue.

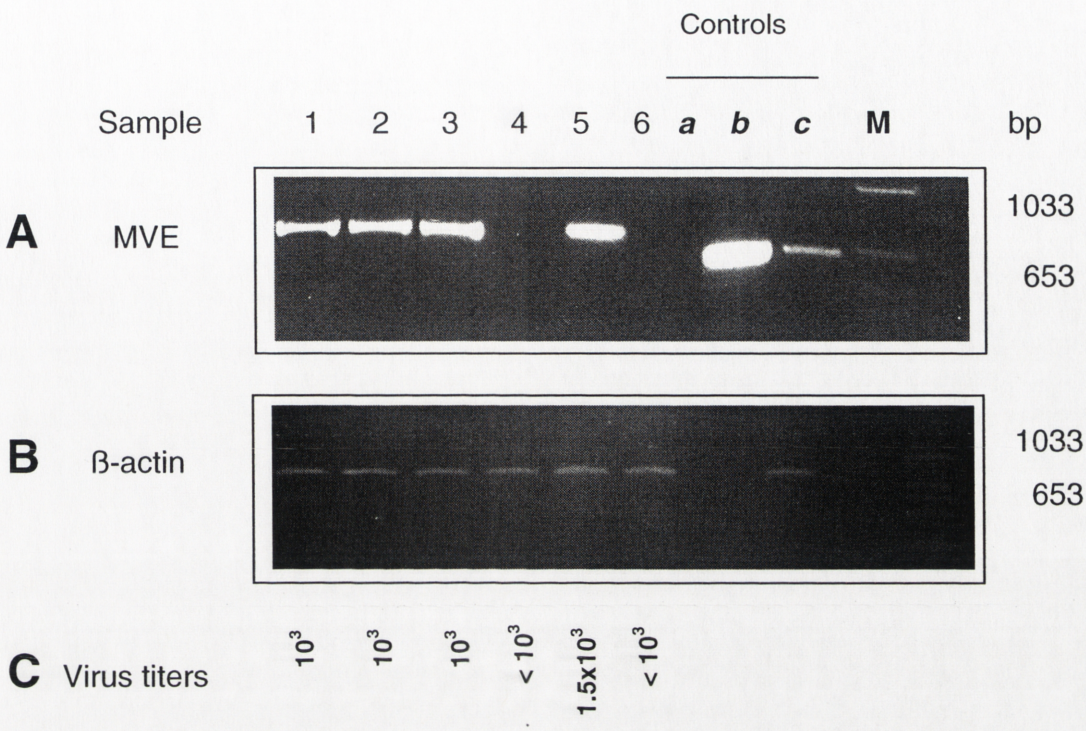

Fig. 3.2 A comparison of the results of RT/PCR and PA of spleens taken from mice that were infected iv with 10^2 PFU of MVE

Spleen samples (1–6), controls (***a–c***) and DNA molecular weight marker (**M**).

A, MVE-specific RT/PCR. Controls: ***a***, reagents control (water); ***b***, PCR amplification of 10^5 RNA copies; ***c***, PCR amplification of 10^2 RNA copies. Products of 717 bp from infected tissues and 616 bp from the positive control were amplified by MVE-specific RT/PCR.

B, ß-actin specific RT/PCR (540 bp). Controls: ***a***, reagents control (water); ***b***, positive control; ***c***, blank.

C, virus titers obtained from infection of Vero cell monolayers, in PFU/g of wet tissue.

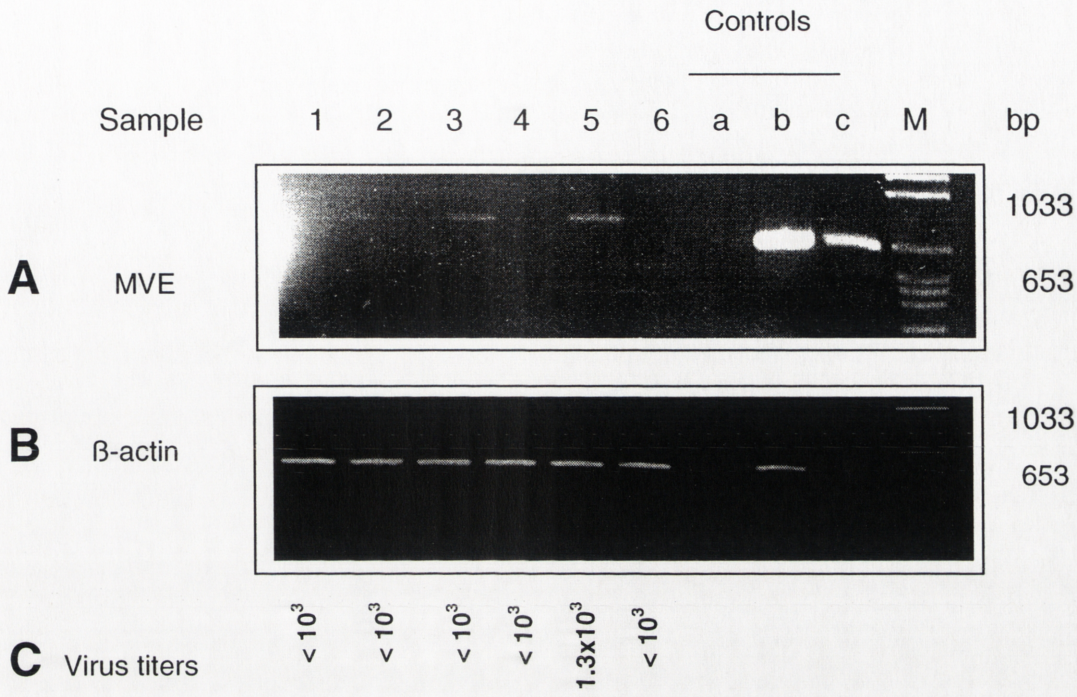

Fig. 3.3 A comparison of the results of RT/PCR and PA of lymph nodes taken from mice that were infected iv with 10^2 PFU of MVE were infected iv with 10^2 PFU of MVE

Lymph node samples (1–6), controls (***a–c***) and DNA molecular weight marker (**M**).

A, MVE-specific RT/PCR. Controls: ***a***, reagents control (water); ***b***, PCR amplification of 10^5 RNA copies; ***c***, PCR amplification of 10^2 RNA copies. Products of 717 bp from infected tissues and 616 bp from the positive control were amplified by MVE-specific RT/PCR.

B, ß-actin specific RT/PCR (540 bp). Controls: ***a***, reagents control (water); ***b***, positive control; ***c***, blank.

C, virus titers obtained from infection of Vero cell monolayers, in PFU/g of wet tissue.

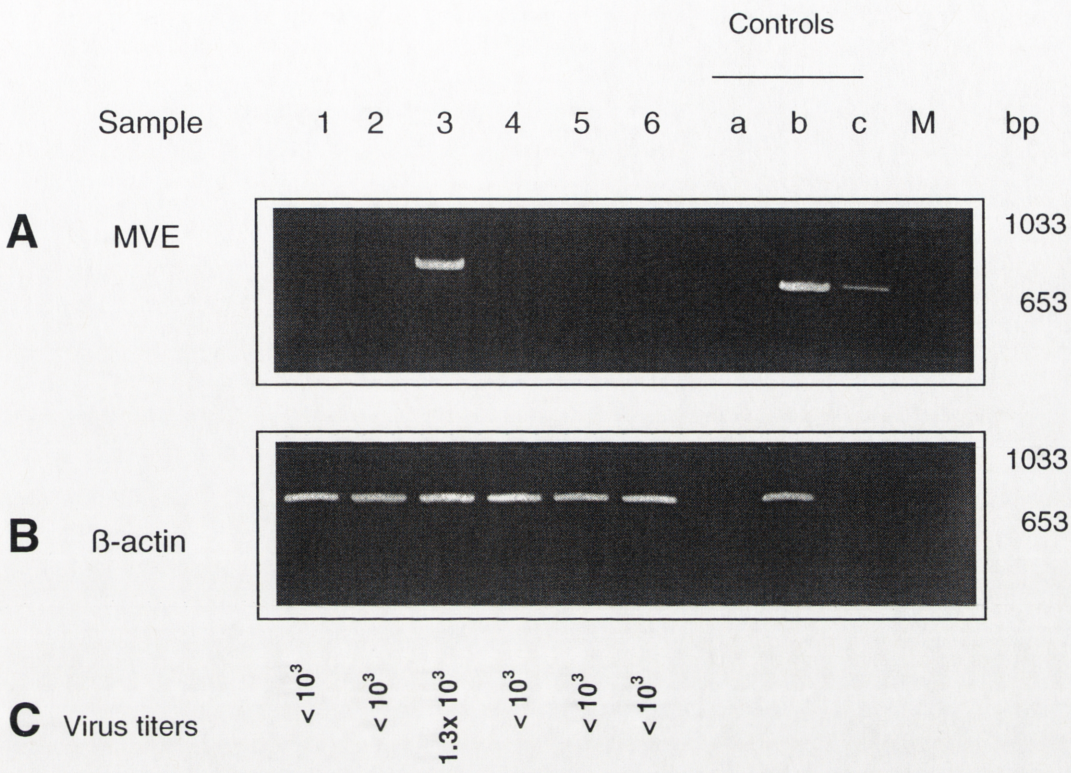

Fig. 3.4 A comparison of the results of RT/PCR and PA of muscle from mice that were infected iv with 10^2 PFU of MVE

Muscle samples (1–6), controls (***a–c***) and DNA molecular weight marker (**M**).

A, MVE-specific RT/PCR. Controls: ***a***, reagents control (water); ***b***, PCR amplification of 10^5 RNA copies; ***c***, PCR amplification of 10^2 RNA copies. Products of 717 bp from infected tissues and 616 bp from the positive control were amplified by MVE-specific RT/PCR.

B, ß-actin specific RT/PCR (540 bp). Controls: ***a***, reagents control (water); ***b***, positive control; ***c***, blank.

C, virus titers obtained from infection of Vero cell monolayers, in PFU/g of wet tissue.

3.3.2 Effect of tissue homogenization on virus infectivity detection

To examine the effect of homogenization of brain, spleen, muscle and liver tissues of uninfected mice on the detection of virus infectivity during plaque titration, several assays were performed. Given the low virus titers detected in most tissues (except in brain), it was important to determine if the difficulty in detecting virus in peripheral organs was because the virus was absent, or because it was present in only small amounts in those organs. Inactivating factors that are released during tissue homogenization could have further reduced such a low presence of virus.

Fifty PFU of virus were mixed with tissue homogenates of brain, muscle, liver and spleen that had been serially diluted by 10-fold (from 10% to 0.01%), and were incubated on ice for an hour prior to plaquing in Vero cell monolayers. I determined to what extent the detection of infectivity was reduced, by comparing the number of plaques that resulted from the combined homogenate-virus inoculum to the number of plaques that were formed by the virus inoculum alone (Table 3.1).

A concentration of 10% of brain homogenate produced an almost complete (94%) inhibition of plaque formation (low numbers and small sizes). Tissue homogenates of 1% and 0.1% reduced the plaques by 70% and 14%, respectively; however, a concentration of 0.01% of brain homogenate had no effect on the number of plaques obtained.

A concentration of 10% (w/v) of spleen homogenate resulted in a 94% inhibition of plaque numbers, while concentrations of 1% and 0.1% reduced plaque numbers by 82% to 18%, respectively. A concentration of 0.01% had no effect on plaque numbers.

Concentrations of 10% and 1% muscle homogenate reduced plaque numbers by 90% and 38%, respectively.

Table 3.1 The percentage of reduction of MVE-infectivity in various concentrations of tissue homogenates[a] tested by PA

Tissue sample[b]	Concentration of homogenate (%)[c]	% reduction[d]
Brain	10	94
	1	70
	0.1	14
	0.01	0
Spleen	10	94
	1	82
	0.1	18
	0.01	0
Muscle	10	90
	1	38
	0.1	22
	0.01	0
Liver	10	100
	1	86
	0.1	24
	0.01	2

[a] Serially diluted tissue suspensions in HBSS-BSA were incubated for 1 h with 50 PFU of virus.

[b] Tissues from non-infected mice were 10% w/v in HBSS-BSA.

[c] Clarified tissue homogenate.

[d] Reduction in % of plaques was:

$$1- \left(\frac{\text{Number of plaques in presence of tissue homogenate}}{\text{Number of plaques in absence of tissue homogenate}} \right) 100\%$$

Liver homogenates produced a significant reduction in plaque numbers on Vero cells. The 10% liver homogenate produced a total inhibition (greater than 99%) of plaque formation, while dilutions of 1%, 0.1% and 0.01% produced a reduction of plaque formation by 86%, 24% and 2%, respectively. The potent effect of liver homogenate on virus infectivity may explain why the results in liver samples are negative when they are tested by plaque assay.

In summary, homogenates of brain, spleen and liver showed a significant inhibition of plaque formation at concentrations greater than 0.1% w/v, and muscle homogenates had the least inhibitory effect on MVE infectivity of the four tissues tested.

3.4 Discussion

In this chapter, I compare two commonly used methods of virus detection in tissues: PA detects infectious virus in tissue culture, whereas RT/PCR amplifies viral RNA. It is important to test aliquots from the same samples using both methods, since different organs may contain factors, such as the amount of RNAse (relevant during RT/PCR, since RNA is the starting material), or infectivity-inhibiting substances (important during PA), that could affect the detection of viral genetic material or infectious virus, respectively. Overall, the results from the RT/PCR and PA methods for tissues such as brain, spleen, and muscle are similar (positive or negative). These results correspond to the ones obtained by Bae et al. (2003), who compared the precision of the plaque assay and real-time PCR in the detection of YF.

There is evidence that flaviviruses have preferential sites of replication in the brain at different stages of the infection. In neuroinvasion experiments of 3-week-old

mice, the olfactory lobe has a higher viral content than the other parts of the brain in the initial stage of invasion. Later in the infection, the structures of the hippocampus have a higher viral content. Virus spreads to most of the brain immediately before death (Monath et al., 1983; Matthews et al., 2000). Thus, given that the virus can be present in different locations within the brain at different stages of the infection, a small sample of the brain may not be representative of the organ. Consequently, to ensure that all the areas of the brain that support virus replication are included, at least one hemisphere of the brain should be homogenized. Therefore, plaque assay seems to be an ideal method for detecting virus in the brain, because a representative sample from the homogenization of half a brain can be processed for tissue titration. Thus, the standard 3 mm^3 of tissue taken for RNA extraction and PCR testing used for other extraneural organs may not be suitable for brain tissue, as such a sample would not be representative for virus replication in all areas of the brain.

Lymph nodes more often gave positive results when they were analyzed by RT/PCR than by PA. The small size of lymph nodes and the adherent characteristics of the tissue were a problem during the PA test because most of the tissue remains on the wire mesh during tissue homogenization, probably along with the virus. Thus, the difficulty in detecting virus in lymph nodes by plaque assay may have been due to technical problems. To avoid this problem and to increase the amount of lymph node tissue, I collected lymph nodes from the axillary, inguinal and cervical areas for my subsequent experiments (see Chapters 4–7). Because MVE was inoculated iv, I was able to use any lymph node, since there were no specific virus-draining lymph nodes.

From the above experiments, one could conclude that the RT/PCR method is more sensitive in detecting virus than the PA method. However, the RT/PCR method's higher detection rate may be because it detects RNA from inactivated virus, as was

previously shown for the human T-lymphotropic virus 1 (Morozov and Weiss, 1999). This would mean that RT/PCR results are not suitable for drawing conclusions about the infectious potential of the virus (Bae et al., 2003). Besides the latter deficiency, the RT/PCR method has several other known limitations (Piatak et al., 1993; Souaze et al., 1996; Deubel, 1997). Firstly, the amount of product obtained by the RT/PCR method does not consistently reflect the amount that is present in the initial target. This is probably because PCR and/or RT have different efficiencies and kinetics that depend upon the abundance of the target, and the presence of various inhibitors (Piatak et al., 1993; Deubel, 1997). Secondly, the comparison of the amount of viral RNA that is amplified from the specimen, to the amount that is amplified from a titrated standard (in separate reactions) is not ideal for quantification (Wang et al., 2000). Thirdly, the process of normalization by co-amplifying a heterologous target (such as actin or ß-globulin) cannot provide a good internal control because there are differences in the abundance of the heterologous target and its priming efficiency (Piatak et al., 1993; Deubel, 1997). Additionally, the RT/PCR method has a higher risk of cross-contamination than the PA method, as its molecular techniques require a number of different steps where contamination can occur, e.g. RNA purification, reverse transcription and PCR. In my study, contamination during the RT/PCR process was only detectable if the reagent control and the positive control had a smaller size than the testing samples. Consequently, because cross contamination between the tissue samples in the RT/PCR process cannot be identified, the possibility of false positive results is increased. Compared to the limitations of RT/PCR, quantification by PA is simple and reliable.

Another factor that needs to be considered when deciding which method is better is the reduction of infectivity that occurs through the release of inhibitory factors during

tissue processing for the PA method. The tissue homogenates that are used during plaque assay drastically reduce virus infectivity when 10% to 1% tissue suspensions are used. If virus titers in the tissues are low, virus detection could become masked by the inactivating reaction that is caused by the factors that are present in the tissues. The content of the MVE in the organs is not affected by these factors when less than 0.01 of homogenates are used (my observations), or 0.5%, as shown by Lee and Lobigs (2002), so virus titers obtained at these dilutions are considered reliable.

The results I present in this study confirm that there is poor MVE replication in peripheral organs tested in 6-week-old mice. This, together with the technical difficulties such as viral RNA degradation and the reduction of virus infectivity by factors that are present in the tissue homogenates, make virus detection in peripheral organs even more difficult. Most of the results given by the two techniques coincide when the same samples are tested. For the purpose of the work in my thesis, I selected the traditional PA as the technique to use in further experiments, as it is a simple method that obtains quantitative results and detects infective virus.

Chapter 4

Pathogenesis of MVE in adult C57Bl/6 wt mice

4.1 Summary

This chapter presents the establishment of a mouse model of encephalitic flavivirus infection that parallels infection in humans. In particular, only a subset of the mice used develops disease. The virus is administered by the intravenous route, which is analogous to the portal of entry in natural infections, and the mice are given a virus dose in the range that would be experienced following the bite of an infectious mosquito.

The results show that mice of 6 weeks of age were susceptible to doses of MVE in the range of 0.1 to 10^5 PFU, which resulted in close to 50% mortality, and that 10-week-old mice were mostly resistant to similar doses of virus. Mice of all ages were susceptible, even to low doses, after ic flaviviral infections. Susceptibility in 6-week-old mice after iv infection was higher than after ip infection. Because females did not show a significant difference in susceptibility compared with males, both genders were suitable for further studies. After iv infections with 10^2, 10^3 and 10^5 PFU, MVE titers in extraneural organs were low or undetectable, despite 100% seroconversion, which confirmed infection. After infection with a high dose of 10^8 PFU that produced 100% mortality, titers in extraneural organs were relatively low, but titers in the brain showed that virus had already reached the target organ at day 2 pi. Titers in the brain further increased to 10^9 PFU/g on day 3 and 4 pi. The rapid neuroinvasion after infection with a high dose indicated that the virus crossed the blood–brain barrier (BBB) and did not need peripheral replication. Histopathology correlated in most of the cases with the level of infection: foci of inflammation and tissue destruction were rare or undetectable in extraneural organs but prominent in brains of moribund mice. *In vitro* studies showed that MVE-specific Tc-cell (H-2^b-restricted) responses were induced.

4.2 Introduction

Mice are relatively good laboratory animals to model flaviviral encephalitis in humans. All members of the JE serocomplex are neurotropic in mice, with clinical and histopathological features of young mice infected with encephalitic flaviviruses similar to those that appear in humans (Monath, 1986). However, the lack of a fully mature immune system in the young mouse model is a limitation to the study of immune responses against flaviviruses. In most of the cases, adult immunocompetent mice are susceptible to flaviviruses only after ic inoculation, a route of infection that does not mimic the natural route of infection via mosquitoes.

Macaques are susceptible to encephalitic flavivirus infection and would probably represent the disease in humans better, but their high costs make them an impractical host model (Markoff, 2000; Monath et al., 2002). In order to fulfill the requirements of an ideal animal model, small adult laboratory animals should present morbidity and mortality after systemic infection (Leyssen et al., 2000). The role of the immune responses in the protection against flavivirus-induced disease has been difficult to demonstrate *in vivo* due to the above limitations.

Previous studies on encephalitic flaviviruses show that humoral immunity is the key to prevent the spread of virus from the blood to the brain (Camenga et al., 1974; Kimura-Kuroda and Yasui, 1988; Schlesinger et al., 1993). Passive transfer of antibodies can protect animals against a lethal flavivirus challenge, including an infection via the ic route, while neutralizing antibodies to the E protein protect them against a homologous flavivirus challenge (Gould et al., 1986; Hawkes et al., 1988; Kimura-Kuroda and Yasui, 1988). However, the kinetics of induction of humoral immunity in a primary flavivirus infection may not be sufficiently rapid to prevent neuroinvasion and encephalitis after peripheral virus growth. Cell-mediated immunity

(CMI) develops with a kinetics that is consistent with the requirement for rapid virus elimination (Gajdosova et al., 1981; Kesson et al., 1987). Flavivirus-immune cytotoxic T (Tc) cells are generally apparent at day 5 pi, and their numbers peak on day 7 pi. The flavivirus NS3 protein contains Tc-cell determinants presented by a number of mouse and human MHC class I restriction elements (Kurane et al., 1989; Kurane et al., 1991; Parrish et al., 1991; Rothman et al., 1993; Lobigs et al., 1994). The role of the Tc cells in recovery from encephalitic flavivirus infection may depend on whether they can prevent virus from spreading into the CNS while their function is directed against virus-infected cells in the periphery. Although virus-specific immune Tc and NK cells have been isolated from brains of flavivirus-infected mice, it is still unclear whether they are necessary for the recovery from encephalitic flavivirus infection, or whether they induce immunopathology, as has been observed in some viral models (Doherty and Zinkernagel, 1974; Moskophidis and Kioussis, 1998), including flaviviral models (Camenga and Nathanson, 1975; Semenov et al., 1975). Thus, the relative contribution of cytotoxic lymphocytes to neuronal destruction remains ambiguous (Nathanson and Cole, 1970; Lad et al., 1993; Andrews et al., 1999).

The work described in this chapter concerns pathogenesis studies of MVE infection of C57Bl/6 wild-type (wt) mice. This study covers the dual aspect of the pathogenesis, namely the ability of the virus to invade the host and inflict pathogenesis, and the ability of the host to clear the infection, given that both death and survival of the hosts are obtained under similar conditions of infection.

4.3 Results

4.3.1 Mortality after MVE infection of C57Bl/6 mice according to age and gender of mice, and infection route and dose of virus

Flaviviruses induce an age-dependent disease in mice. Because it was of interest to establish at which maximum age mice are still susceptible to MVE infection, I tested mice of 6 and 10 weeks of age (Table 4.1 A). I also tested if the mice showed a gender difference in susceptibility (Table 4.1 B), and tested which route of infection leads to a higher susceptibility when the virus is inoculated systemically (the ip or iv route). I used the ic route of inoculation to find out what the mortality time would be once the virus is present in the brain (Table 4.1 C). Once I established the maximum age for susceptibility with no apparent gender difference, and the route of the infection, I tested different iv doses — ranging from 0.1 to 10^8 PFU — in 6-week-old mice (Table 4.1 D). I recorded morbidity and mortality daily until day 21 pi, when mice either had asymptomatic infection, recovered from a mild clinical disease, or died.

Age-dependent susceptibility of C57Bl/6 mice to infection with MVE

Mice of 6 and 10 weeks of age received MVE via the iv route. Each group was injected with doses of either 10^2 or 10^8 PFU. A dose of 10^2 PFU killed 46% of the 6-week-old mice, but did not kill any of the 10-week-old mice. The higher dose of 10^8 PFU killed all the 6-week-old mice ($P = 0.11$) at day 5.5 pi and 80% of the 10-week-old mice at day 6.8 pi (Table 4.1 A). I chose to continue with 6-week-old mice for further work, since they were optimally susceptible to MVE and had a mortality of 50% after infection with the low dose (although they had a fully developed immune system).

Effect of gender on mortality of 6-week-old C57Bl/6 mice after MVE infection

The effect of gender was tested to investigate whether possible behavioral differences, including stress (e.g. males showing a fighting tendency) and weight differences (males were on average three grams heavier than females of the same age), contributed to mortality when the conditions of infection were similar (Table 4.1 B). After I infected a group of 14 males and 15 females with a dose of 10^2 PFU iv, I observed a mortality of 43% and 40%, respectively, a difference that was not statistically significant ($P = 1.0$). The higher average weight of males, and the lower amount of virus per unit of body weight in males relative to females did not lead to an increased resistance to infection or a statistical difference in the ATD ($P = 0.40$).

Effect of route of infection on mortality of 6-week-old C57Bl/6 mice

The ip, iv and ic routes of infection were compared to determine their influence on the pathogenicity of MVE (Table 4.1 C). I compared the ip with the iv route of infection after injecting mice with doses of 10^3, 10^5 and 10^8 PFU of virus. Of those mice that were injected with doses of 10^3 and 10^5 PFU, the mice that were injected iv consistently showed higher fatalities than the mice that were injected ip, although there was no statistically significant difference. Of those mice that were infected with a dose of 10^8 PFU of MVE, the mice that were injected ip showed 70% mortality, but the mice that were injected iv yielded no survivors; a difference which was statistically significant ($P = 0.03$). Intracerebral inoculation of a low dose of 10 PFU of virus produced an acute disease that was similar to the disease after iv injection of 10^8 PFU, resulting in both cases in a 100% mortality between days 5 to 7 pi. Mice injected ic with

10 PFU of virus died after an average of 6.4 days pi, i.e. slightly later than mice injected iv with 10^8 PFU, which died after an average of 5.5 days pi. Thus, it would appear that once virus enters the CNS, either by direct injection into the brain or after iv administration of 10^8 PFU, death is rapid and inevitable.

Dose-response of MVE in C57Bl/6 mice

Peripheral inoculation with viral doses in the range of 0.1 to 10^5 PFU of MVE by the ip or iv route killed approximately 50% of the animals, but characteristically failed to produce a significant dose-dependent increase in mortality, whereas a high virus dose of 10^8 PFU by the iv route consistently resulted in 100% death (Table 4.1 D). An iv-injected virus dose of 0.1 PFU resulted in 59% mortality, while an iv injection of 10^2 PFU resulted in 46% mortality ($P = 0.26$). This slight inverse correlation between virus dose and percentage of mortality was also observed in MVE-infected mice by Boyle (1979). I observed two distinct mechanisms for the average time to death (ATD): mice injected with 10^8 PFU of MVE died at 5–6 days pi, whereas mice injected with the lower doses died from day 9 to 13 pi (Table 4.1 D). This pattern has been also observed for the encephalitic flavivirus WNV (Wang et al., 2003).

Animals that succumbed to MVE infections showed similar clinical symptoms at one to two days prior to death. This observation, in addition to observing similar virus titers in the brains of moribund mice from groups that were peripherally infected with the high or lower doses, suggests that in both cases viral encephalitis was the cause of death.

Table 4.1 Mortality of MVE-infected C57Bl/6 wt mice according to mouse age and gender, infection route and dose

A. Age response

Age (w)	Dose (PFU)	N[a]	% Mortality	ATD ± SEM[b]
10	10^2	10	0	--
10	10^8	10	80	6.8 ± 0.5
6	10^2	70[c]	46	11.6 ± 0.4
6	10^8	19[c]	100	5.5 ± 0.2

B. Gender response

Gender	Average weight (g)	N[a]	% Mortality	ATD ± SEM[b]
male	20.2	14	43	11.9 ± 0.8
female	17.0	15	40	10.7 ± 1.2

C. Route of infection

Route	Dose (PFU)	N[a]	% Mortality	ATD ± SEM[b]
iv	10^3	9[c]	56	12.0 ± 0.8
iv	10^5	12[c]	50	10.3 ± 0.7
iv	10^8	19[c]	100	5.5 ± 0.2
ip[d]	10^3	58	40	13.7 na
ip[d]	10^5	32	38	11.8 na
ip[d]	10^8	10	70	8.6 na
ic	10	5	100	6.4 ± 0.5

D. Dose response

Dose (PFU)	Na	% Mortality	ATD ± SEMb
0.1	27	59	10.2 ± 0.7
10^2	70[c]	46	11.6 ± 0.4
10^3	9[c]	56	12.0 ± 0.8
10^5	12[c]	50	10.3 ± 0.7
10^8	19[c]	100	5.5 ± 0.2

[a] Total number of mice infected per group. Morbidity and mortality were recorded daily. Surviving mice were monitored for 21 days. All mice were 6 weeks old, except in A. Mixed groups of males and females, except in B. Infection of mice by the iv route, unless otherwise specified (C).

[b] The average time to death (ATD) in days ± standard error of the mean (±SEM).

[c] The groups of mice in D are repeated in A and C.

[d] Mortality studies after ip infection, performed by Ms G. Colombage (personal communication); no statistics available (na).

4.3.2 Kinetics of replication of MVE in tissues from infected 6-week-old C57Bl/6 mice

MVE growth in extraneural tissues

The virus load in tissue homogenates was studied by plaque assay to determine which extraneural organs (ovaries, serum, blood cells, kidneys, spleen, muscle, lymph nodes and liver) supported virus replication, and to define the kinetics of virus growth before the virus reaches the CNS. Liver, kidneys, ovaries and blood cells did not show detectable virus titers even after infection with a high dose of 10^8 PFU (tested by both plaque assay and RT/PCR, data not shown) and were not further studied. Using plaque assay, I tested spleen, muscle and lymph node samples from mice infected iv with 10^2 PFU (Fig. 4.1) and 10^5 PFU (Fig. 4.2) of virus, for the presence of virus at two-day intervals, and I tested samples of mice infected with 10^8 PFU (Fig. 4.3) of virus at one-day intervals. Serum was also collected after infection with 10^8 PFU.

Consistent with earlier studies (MacDonald, 1952), I found that MVE grows poorly in extraneural tissues of mice of this age. After inoculating mice with 10^2 PFU iv, I did not detect any virus in their extraneural organs (spleen, lymph nodes and muscle) (Fig. 4.1), although the mice all seroconverted (Table 4.2). Infection with 10^5 PFU resulted in barely detectable virus titers in the spleen at day 4 pi, clearing of the virus by day 6 pi (Fig. 4.2).

Intravenous inoculation of 10^8 PFU of virus resulted in virus replication in the spleen, muscle and lymph nodes (Fig. 4.3), although virus titers were low, and virus was not detected in liver (data not shown). Virus titers in the spleen were the highest on day 2 pi and were absent on day 4 pi in most animals (Fig. 4.3 B). Since virus titers seemed to decrease with time in the spleen, there was probably minimal or no detectable viral replication in that organ. Growth of MVE in muscle occurred in a few mice on

days 2, 3, and 4 pi, and virus titers were slightly higher ($2x10^4$ PFU/g), and present in more mice, on the later collection days (Fig 4.3 C). This result could be an indication that muscle, as well as brain, may support virus replication, instead of only brain, as has been reported by other studies with MVE (Murphy et al., 1968; Hase et al., 1990; McMinn et al., 1996; Andrews et al., 1999). I also collected and pooled mesenteric and inguinal lymph nodes for virus titration, and found that virus titers were similar to those of the spleen (Fig 4.3 E). Low virus titers were detectable in serum at day 1 pi, ranging only from 10^2 to 10^3 PFU/ml, while the limit of detection was 10^2 PFU/ml (Fig. 4.3 D). The lack of virus detection in serum at later time points was an indication that the virus I detected in the tissues collected from day 2 pi was not a carry-over from the blood. This indicates that adult mice show no detectable viremia, and that they are therefore similar to human cases (Monath, 1996).

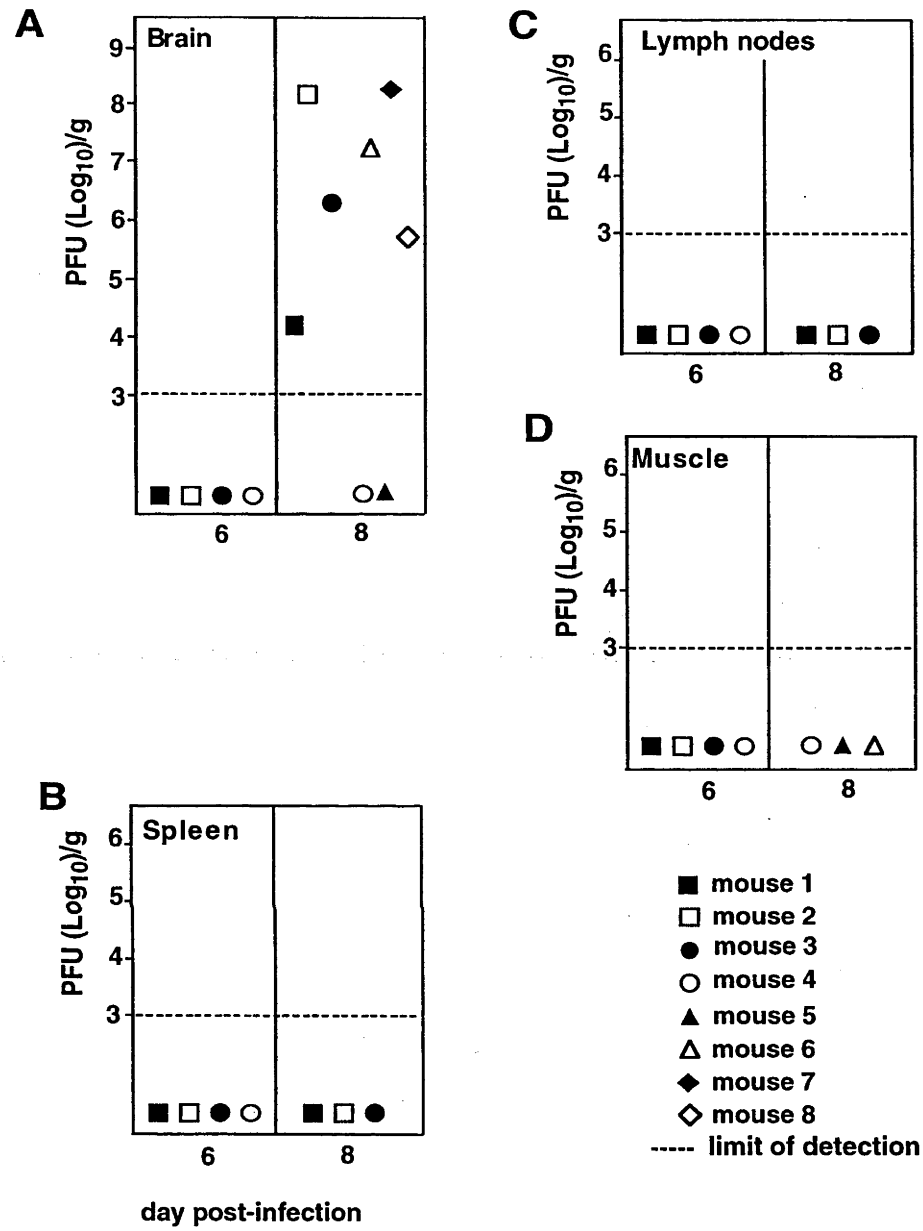

Fig. 4.1 Growth of MVE in tissues of C57Bl/6 wt mice. Six-week-old mice were infected with 10^2 PFU of MVE iv. At the indicated times, animals were sacrificed, and virus titers in the brain (**A**), spleen (**B**), lymph nodes (**C**) and muscle (**D**) were determined by plaque assay. The lower limit of detection of virus titers was 1 x 10^3 PFU/g of wet tissue and is indicated by the interrupted line. The different symbols represent tissues of individual mice (mouse 1–8).

Kinetics of neuroinvasion and growth of MVE in the brain of wt mice

The time to neuroinvasion and the level of virus growth in the brain were tested in groups of mice that were infected iv with low (10^2 PFU), medium (10^5 PFU), or high (10^8 PFU) doses of MVE. Brains from most of the mice (six out of eight) infected with the low dose only showed detectable virus titers on day 8, with values that ranged from 10^3 to 10^8 PFU/g (Fig 4.1 A). The increase of 3 logs (from 10^2 to 10^5 PFU/g) in the viral inoculum did not result in an increase in titer values (i.e. an earlier detection) in the brain. On the contrary, none of the brains from the mice that were infected with the medium dose had detectable virus titers at any of the collection time points (Fig. 4.2 A). However, an average of 50% mortality at day 10, after infection with 10^5 PFU (which was similar to mortality after infection with 10^2 PFU), indicated that neuroinvasion occurred, but probably after the last brain sample was collected (day 8 pi). Mice infected with a high virus dose of 10^8 PFU had detectable virus titers in the brain, which ranged from 10^3 to 10^5 PFU/g on day 2 pi, and increased to a range of 10^7 to 10^9 PFU/g on day 4 pi (Fig. 4.3 A).

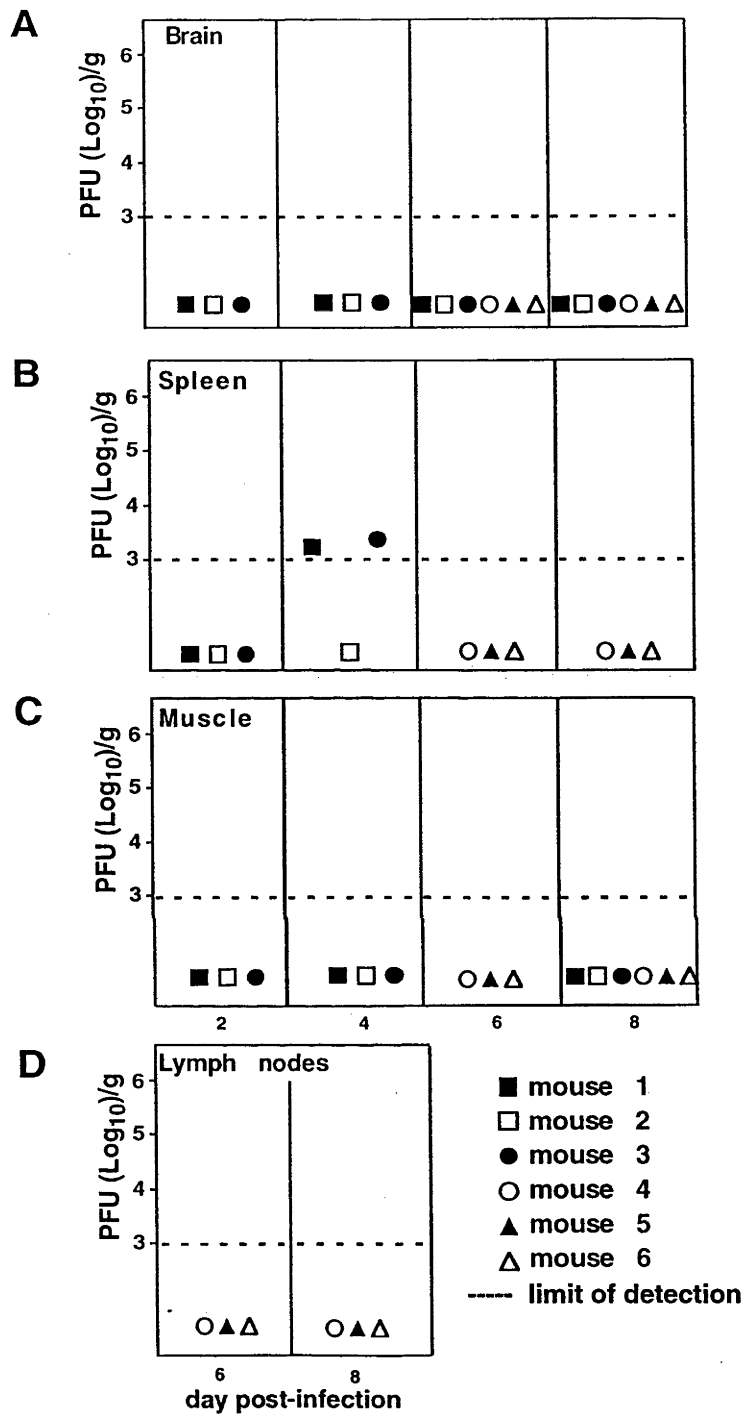

Fig. 4.2 Growth of MVE in tissues of C57Bl/6 wt mice. Six-week-old mice were infected with 10^5 PFU of MVE iv. At the indicated times, animals were sacrificed, and virus titers in the brain (**A**), spleen (**B**), muscle (**C**) and lymph nodes (**D**) were determined by plaque assay. The lower limit of detection of virus titers was 1 x 10^3 PFU/g of wet tissue, and is indicated by the interrupted line.

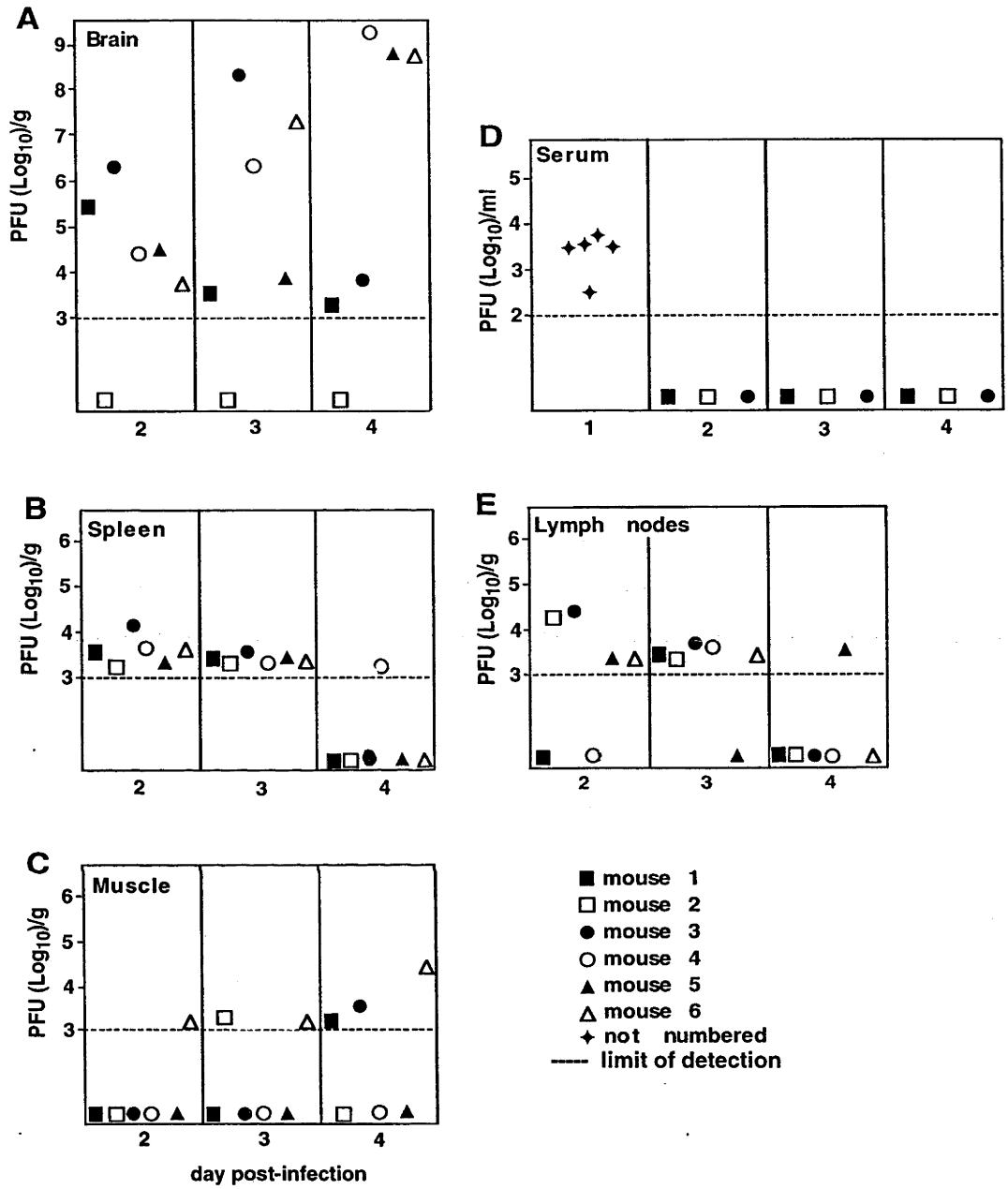

Fig. 4.3 Growth of MVE in tissues of C57Bl/6 wt mice. Six-week-old mice were infected with 10^8 PFU of MVE iv. At the indicated times, animals were sacrificed, and virus titers in the brain (**A**), spleen (**B**), muscle (**C**) serum (**D**) and lymph nodes (**E**) were determined by plaque assay. The lower limit of detection of virus titers was 1 x 10^3 PFU/g of wet tissue and 10^2 PFU/ml for serum, and is indicated by the interrupted line. The different symbols represent tissues of individual mice (mouse 1–6).

4.3.3 MVE-specific antibody response

The Ab response was measured using ELISA. Serum from 6-week-old mice that were iv infected with 0.1 PFU of virus were tested at day 10 and 22 pi; mice infected with 10^2 PFU were tested at day 10, 22, and 27 pi; and mice infected with 10^5 PFU were tested at day 5, 10, 15 and 21 pi. I also tested older mice of 10 weeks of age for Ab production at day 25 pi, after infection with 10^5 PFU. Antibody titers and the incidence of seroconversion were important because they allowed me: 1) to verify that infection of mice with the doses of MVE used in the pathogenesis and virulence assays resulted in infection of extraneural tissues, and 2) to test whether differences in the magnitude of the humoral response against different doses of MVE were detectable. The sera from all infected animals were reactive against MVE antigen (Table 4.2). Because a single vaccination of mice with 10^2 PFU of UV-inactivated MVE does not induce MVE-specific antibodies detectable by ELISA (data not shown), the 100% seroconversion indicates that all the mice had been effectively infected and that virus growth in peripheral tissues had occurred.

Mice infected with the low dose showed considerable Ab levels, with mean values of 3.7 Log_{10} and 4.3 Log_{10} on day 10 and 22 pi, respectively. This indicates that despite the low dose of 0.1 PFU of virus, the dose was high enough to induce 100% seroconversion. Sera from mice, infected with 10^2 PFU of virus iv, collected on days 10, 22 and 27 pi, showed similar levels of Ab (Log_{10} of 3.7, 3.9 and 4.3, respectively). Mice infected with 10^5 PFU showed Ab titers from early on (day 5 pi) — in accordance with studies made by McMinn et al. (1996) — and gradually increased to Log_{10} of 4.0 by day 21 pi. The increase in injected virus from 0.1 to 10^2 PFU, and even 10^5 PFU, did not cause a significant difference in Ab production. In general, antibody titers increased

gradually until day 21 pi, when antibody production is considered to be at a plateau level (Monath, 1986).

Ab levels from 10-week-old mice infected with 10^5 PFU ranged from 3.5 to 4.4 Log_{10}, at day 25 pi, with a mean value of 3.9 Log_{10} (data not shown). This result suggests that the increase in age from six to ten weeks does not necessarily bring about an increase in the capacity for Ab production (although, I did not measure the level of neutralizing Ab). Six-week-old mice had a mean Ab production of 4.5 Log_{10} on day 21 pi, and this value was not statistically different from the levels produced by 10-week-old mice.

Table 4.2 MVE-specific Ab production in 6-week-old C57Bl/6 wt mice

Dose (PFU)[a]	No./ group[b]	% Seroconversion	Day pi	Mean Ab titer, Log_{10} (range)[c]
0.1	6	100	10	3.7 (2.6-4.1)
0.1	4	100	22	4.3 (3.8-4.7)
10^2	6	100	10	3.7 (2.6-4.1)
10^2	6	100	22	3.9 (2.9-4.7)
10^2	5	100	27	4.3 (3.8-4.7)
10^5	6	100	5	2.5 (2.0-2.9)
10^5	7	100	10	3.4 (3.2-3.5)
10^5	7	100	15	3.7 (3.5-3.8)
10^5	12	100	21	4.0 (3.5-4.7)

[a] Intravenous MVE inoculation.

[b] Number of mice tested for MVE-specific antibody titers determined by ELISA.

[c] The endpoints were calculated as described in Materials and Methods, Chapter 2.

4.3.4 MVE-immune Tc-cell response in C57Bl/6 mice

To verify that MVE-specific Tc-cell responses are induced in C57Bl/6 mice (Fig. 4.4), splenocytes were obtained from groups of four mice that had been infected ip with 10^6 PFU of MVE 10 days previously. I tested secondary, *in vitro* stimulated, MVE immune splenocytes against MVE-infected and uninfected 2R (K^k, D^b) and 5R (K^b, D^d) target cells using ^{51}Cr release assays. Figure 4.4 A shows that D^b- and K^b-restricted anti-MVE Tc-cell responses are elicited in MVE-infected C57Bl/6 mice, with the former having the stronger response. Given that specific killing of H-2-mismatched MVE-infected target cells (L929, H-2^K) by the MVE-immune effector cells from C57Bl/6 mice was not observed, the D^b- and K^b-restricted lysis was Tc-cell mediated (data not shown).

To identify the MVE-specific antigenic determinant(s) that are recognized by H-2^b-restricted Tc cells, I infected 2R (K^k, D^b) and 5R (K^b, D^d) target cells with the VV recombinant viruses (VV-STR, VV-prM-E, VV-prM, VV-E, VV-NS, VV-NS1-NS3, and control VV-TK$^-$) encoding overlapping regions of the MVE polyprotein (Fig. 4.4 C). The 2R target cells infected with recombinants VV-STR, VVprM-E, or VV-prM, were lysed by MVE-immune Tc cells, whereas the target cells infected with the constructs VV-E, VV-NS, or VV-NS1-NS3 or with the control virus, VV-TK$^-$, were not recognized.

Accordingly, the D^b-restricted response was directed against a determinant that was derived from the viral prM protein (Fig. 4.4 B). The VV-immune Tc cells recognized all the VV-infected target cells, but not the uninfected cells (Fig. 4.4 B). The K^b-restricted anti-MVE Tc-cell response was directed against a determinant(s) that was derived from the non-structural polyprotein region down-stream from NS2B (data not shown).

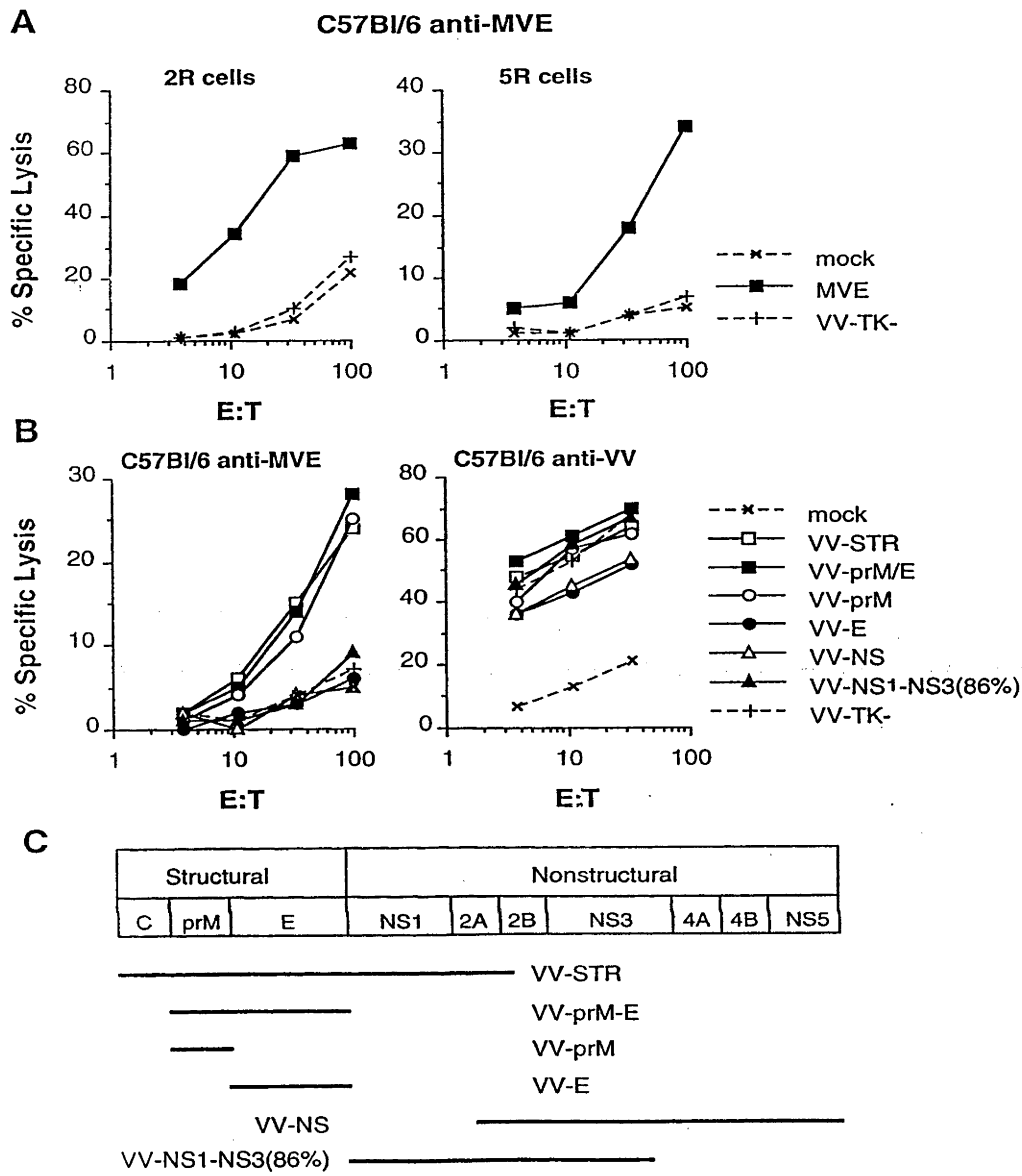

Fig. 4.4 MVE-infection of C57Bl/6 wt mice elicits H-2^b-restricted anti-viral Tc-cell responses. **A.** Lysis of ^{51}Cr-labelled 2R (K^k, D^b) and 5R (K^b, D^d) cells infected with MVE, VV-TK- or left uninfected by secondary *in-vitro*-stimulated MVE-immune Tc cells from wt mice. **B.** Lysis of ^{51}Cr-labelled 2R (K^k, D^b) and 5R (K^b, D^d) cells from mice that were infected with constructs VV-MVE; mock infected mice and VV-TK- as controls. **C.** Schematic representation of MVE polyprotein fragments expressed via VV recombinants. The MVE polyprotein is shown at the top. Regions encoded by the VV recombinants used in this study are shown below.

4.3.5 Histopathology

To further analyze the disease progression of mice infected iv with 10^2 to 10^8 PFU, I did kinetic studies to follow the virus load and histopathology. Pathology and virus titer studies of brain, spleen, muscle, lymph nodes, kidney and liver of mice infected iv with 10^2 and 10^5 PFU of virus showed that hardly any virus could be detected in the different tissues, and that cell infiltration or histopathology were rare (data not shown). Results that were obtained from mice that had been infected iv with a higher dose of 10^8 PFU are shown in Table 4.3. Between days 2 and 4 pi, I daily collected brains and spleens from three individual mice in each group. From day 2 pi, virus titers in all the brains were above the detection limit (>10^3 PFU/g), and increased to up to 10^9 PFU/g on day four. Mr Thomas Steele (Max Plank Institute, Freiburg, Germany) observed perivascular infiltration (pvi) in brain samples only on the last days of collection (in one brain on day 3 and in all three brains on day 4) (Fig. 4.3). This early manifestation of pathology on day 3 and 4 pi, visible as cell infiltration in the brain (the type of the cells that infiltrated was not identified), suggests that innate immunity was involved in the defense against acute MVE infection. Contrary to the results of virus load in the brain, titers in the spleen reduced with time pi. This correlated with splenic histology studies, which showed a gradual reduction in pathology. At day 2 pi spleens showed reactivity with the development of secondary follicles, and an enlarged red pulp and a white pulp. From day three to day four, spleens showed only reactivity, consistent with the reduction observed in the virus titers.

Table 4.3 Histopathology of brain and spleen from wt mice infected iv with 10^8 PFU of virus

	Brain		Spleen	
Day pi	**Histology**	**Plaque assay**	**Histology**	**Plaque assay**
2	normal	$1.5x10^4$	reactive, red pulp enlarged, white pulp with secondary follicles	$5x10^3$
2	normal	$2x10^4$	reactive, red pulp enlarged, white pulp with secondary follicles	$2x10^3$
2	normal	$4.5x10^3$	red pulp enlarged	$5x10^3$
3	normal	$2.2x10^6$	reactive	$1.5x10^3$
3	normal	$9.5x10^3$	reactive	$4x10^3$
3	extensive pvi	$2.2x10^7$	reactive	$3x10^3$
4	extensive pvi	$2.7x10^9$	reactive	$1.5x10^3$
4	extensive pvi	$6x10^8$	reactive	$<10^3$
4	mainly pvi	$5.4x10^8$	reactive	$<10^3$

normal: no histological lesions, similar to mock-infected mice (data not shown).
pvi: perivascular infiltration.
reactive: reaction to antigens with the development of secondary follicles.

4.4 Discussion

The lack of an effective immunocompetent animal model has been a major obstacle in attempts to define the role of humoral and cellular immune responses during flaviviral infections (Hall et al., 1996). The typical animal model for flaviviruses has been the 3-week-old mouse, which shows nearly 100% of disease incidence. This model is poorly suitable to reproduce the disease of humans, in the sense that interpretation of the level of immune protection or the level of immunopathology during infection is difficult because young mice have an underdeveloped immune system. Additionally, the BBB in those mice is not yet fully mature. Thus, a detailed study of the pathogenesis of flaviviruses in immunologically mature adult mice is warranted. In this chapter, I have described a model for the study of the pathogenesis of encephalitic flaviviruses using 6-week-old C57Bl/6 wt mice.

Pathogenesis studies involve a pathogen–host relationship under controlled environmental conditions (Oldstone, 1990). In my MVE pathogenesis study, I considered the following aims with regard to the pathogen: 1) to identify how the virulence patterns change according to mouse age and gender, and infection dose and route, and 2) to establish the tissues in which MVE amplification occurs before the virus reaches the target organ (brain). The host factors that are important in defining the mechanisms of protection are: 1) the humoral immune response, which I studied by determining Ab titers at different doses and times pi; and 2) the T-cell immune response, which I measured by testing for the level of *in vitro* cytotoxicity of immune splenocytes to MVE-infected target cells and defining the viral epitopes for Tc cells. Histopathology of brain and spleen was also recorded.

Immunocompetent mice of 6 and 10 weeks old were used for MVE pathogenesis experiments. The10-week-old mice have an increased resistance over the 6-week-old

mice. Thus, the 6-week-old mice were used for further experiments because they are fully immunocompetent and still susceptible to MVE infection after inoculation with the various doses of virus. I did not find gender differences in susceptibility to MVE infection. This finding corresponds to that of Wang et al. (2003), who did studies in 6-week-old mice infected with WNV.

A similar dose of virus, but inoculated by a different route, may result in a different manifestation of the clinical disease (Grossberg and Scherer, 1966). In the case of MVE, infection by the iv route slightly increased the level of susceptibility, compared with infection by the ip route (when a similar dose of virus is used). I therefore used the iv route in most of the subsequent studies. Additionally, the iv injection is also comparable to the portal entry in natural infections, which occurs via the bite of infected hematophagous insects. Although the ic route of infection does not represent the natural way of infection, I used it to define the onset of clinical signs once the virus is in the CNS.

Mortality did not occur in a dose-dependent manner after iv inoculation of virus doses that ranged from 0.1 to 10^5 PFU (all of which produced close to 50% mortality around day 11 pi). The susceptibility of mice to a small dose of virus, such as 0.1 PFU, can help to explain how a small amount of virus that is carried by a mosquito can be enough to produce disease and mortality in a vertebrate host. It is not unusual to find that the highest dilution of virus (0.1 PFU) produced a slightly higher mortality than the higher doses of 10^2, 10^3 and 10^5 PFU. A similar phenomenon has been observed for MVE by Boyle (1979) and for WNV, another encephalitic flavivirus (Wang et al., 2003), and for LCMV (Lehmann-Grube, 1969). This inverse correlation between dose and mortality may be due to the fact that a low dose may not trigger a sufficiently strong immune response to reduce viral replication before there is neuroinvasion and death.

The fact that it was more common to find virus in tissues after infection with 10^2 PFU than after infection with 10^5 PFU, supports this interpretation, although lower levels of immune response were not revealed under the assay conditions of this study.

A dose of 10^8 PFU iv and 10 PFU ic both produced 100% mortality around day six. The fact that the mice injected iv with 10^8 PFU died at a similar time as mice injected ic, combined with the fact that virus was detected from day two onward (first day of collection) in the brain, suggests that the virus enters directly from the blood stream into the CNS, and probably without the need of peripheral replication.

The fact that a balance can develop between the resistance and the mortality in the mouse groups infected with low to medium doses — which resulted in close to 50% mortality — permits the study of the two aspects of pathogenesis: 1) the capacity of the host to defend itself from infection, and 2) the ability of viruses to produce disease. Infection with a high dose of 10^8 PFU caused an acute pattern of disease where, despite full immunocompetence, all the mice succumbed to MVE infection. Animals that were given high or low doses of MVE show similar clinical symptoms at 1–2 days prior to death and have, in addition, similar virus titers in the brains when moribund. This suggests that viral encephalitis is the cause of death, whether by direct infection of the brain or by peripheral infection.

Mice that were peripherally inoculated with doses of 0.1 to 10^5 PFU of MVE had a time to death of 11 days pi on average, while mice that were peripherally inoculated with a high dose had a mean survival time of 5.5 days pi and mice that were ic inoculated survived 6.4 days. The longer time to death in mice infected iv with the lower virus doses suggests that peripheral virus replication may be required prior to neuroinvasion. However, a 10^6-fold difference (from 0.1 to 10^5 PFU) in the amount of initial virus inoculum did not result in any significant change to the time to death. This

suggests that viral growth in the periphery may not be the only factor that determines the time to death following infection.

In addition to mortality, another important aspect for the study of the pathogenesis of a neurotropic virus is its distribution in the tissues. Extraneural organs from 6-week-old mice were found to be largely refractory to MVE replication (virus was hard to find by plaque assay and by RT/PCR), which is consistent with other studies (MacDonald, 1952; Huang, 1963; Monath et al., 1983; McMinn et al., 1996) where MVE was found to grow poorly in tissues of mice that are older than 3 weeks. After infection of 10^2 PFU iv, and close to the time when clinical signs start to appear (day 8 pi), virus growth was only detected in the brain tissues of approximately half the mice. When mice were inoculated with 10^5 PFU, virus was barely detectable in the spleen at day four, and was not detectable in any of the other organs, although all mice had seroconverted. This indicates that a productive infection took place.

The viremia that was detected in mice only one day after infection with 10^8 PFU could be caused by a remnant from the inoculated virus the day before, and not a result of virus replication, because the virus is rapidly removed from the circulation by the reticuloendothelial system (Lee and Lobigs, 2002). Solid organs such as spleen, muscle and lymph nodes showed virus titers that were marginally above the detection limit of 10^3 PFU/g, despite inoculation with a high virus dose. Virus titers in the spleen were higher at early time pi, which may be explained by the fact that the spleen is the organ that collects the antigen from the circulation (Janeway, 1999). The fact that the virus was injected iv presumably facilitated the presence of MVE in the spleen at early times pi. In contrast to the gradual decrease of virus load in the spleen, I observed a moderate increase of MVE in the muscle over time. It is of interest to note that MVE is considered to replicate only in nerve tissue (McMinn et al., 1996). One possible

explanation for the gradual increase of titer values in the muscle could be that, since MVE is a primarily neurotropic virus, it replicates in the peripheral nerve tissue of the leg muscle sample. Because I did not attempt to separate nerve and muscle, the question of whether the virus had replicated in muscle or nerve tissue, remains unanswered.

Histopathology was present in most of the tissues of MVE-infected mice that showed virus titers. Histopathological results indicated that the levels of inflammation, necrosis and perivascular infiltration associated with neurological and splenic infection were more evident in tissues of mice that are infected with the high dose of 10^8 PFU. The increase in brain titers correlated with an increase in the level of histopathology. The inverse situation occurred in spleen tissues, where a decrease in histopathology was found, consistent with virus distribution.

It has been observed that ic inoculation of JEV results in few inflammatory changes, and that there is a rapidly fatal infection in which mice may die before any appreciable host immunological response can take place in the brain (Hase et al., 1990). In contrast, my experiments showed that when a high dose of MVE is injected iv, it is accompanied by the recruitment of inflammatory-immunological elements from the periphery to the brain. This result is phenotypically very different from the results of the localized ic route of infection. The inflammatory reaction I observed in the brain after peripheral infection of 10^8 PFU may have had a deleterious effect, and could have accounted for the reduced AMT of approximately one day in comparison to mortality after ic inoculation (days 5.5 and 6.4 respectively). Similar levels of virus (~10^9 PFU/g) were detected near the time of death, irrespective of whether mice were injected iv or ic.

The MVE-specific antibody response showed no significant dose-dependent difference in the magnitude of the Ab response within the dose range of 0.1 to 10^5 PFU. As little as 0.1 PFU induces Ab production from as early as day 5 pi (the earliest testing

time), consistent with studies by McMinn et al. (1996) where anti-MVE antibody is found as early as day 4 pi, and as late as day 31 pi. The 100% seroconversion found in my experiments indicates that there was an effective infection and possibly a partial Ab protection.

Age was not a significant factor in the Ab production as occurs for mortality. Mice of 10 weeks of age did not show increased Ab levels compared to the 6-week-old mice. The similarity in seroconversion between 6- and 10-week-old mice indicates that the factor that leads to an increase in resistance to MVE infection in the older mice could be caused by something different than Ab protection. However, because I did not use Ab in neutralization studies, I could not establish the level of protection that antibodies may have offered.

My initial *in vitro* studies of the T-cell-mediated immunity by assays of cytotoxicity on spleens from MVE-primed mice (to measure the level of target-cell killing of MVE-infected cells) confirm that wt mice mount an H-2^b-restricted Tc-cell response against MVE. Additionally, the mapping experiments indicate that the K^b-restricted response is directed against a determinant that is derived from the viral prM protein. The strong D^b-restricted anti-MVE Tc-cell response, directed against determinants derived from the non-structural polyprotein region downstream of NS2B (data not shown), raises the question whether it is responsible for the lethal encephalitis in MVE-infected mice, as is the case in the well-studied LCMV mouse-model.

To conclude, in this chapter, general parameters of MVE pathogenesis, such as mortality, virus distribution in mouse tissues, serology, Tc-cell reactivity and histology have been clarified for mice that are still susceptible to MVE infection but are already immunologically mature. Therefore, this immunocompetent mouse model can be used

to study the role of specific immune mechanisms involved in the pathogenesis of MVE infection.

Chapter 5

MVE infection of RAG-1-/- and β_2-M-/- mice, and passive transfer of MVE-immune splenocytes suggest that T cells contribute to pathology and that B cells are indispensable for recovery from infection

5.1 Summary

In this chapter, subtractive (MVE infection of mice with a deficiency in effector cells) and additive (passive transfer of MVE-immune effector T and B cells to MVE-infected wt mice) studies were performed in 6-week-old animals to provide information on the role of B and T cells during the pathogenesis of encephalitic flaviviral disease. The RAG-1-/- mice (B- and T-cell deficient) showed increased susceptibility to MVE infection, compared with wt mice, probably due to the lack of antibody-mediated protection. Conversely, ß2-M-/- mice (that lack expression of MHC class I and hence are $CD8^+$ T-cell deficient) showed increased resistance to the MVE infection, suggesting a deleterious role of Tc cells. Passive transfer of MVE-primed B cells to MVE-infected wt mice resulted in complete protection from the encephalitic disease. In contrast, mortality was slightly higher in infected wt mice that received MVE-primed T cells, indicating that these cells do not play a protective role during MVE infection and that they may even contribute to pathogenesis.

5.2 Introduction

The immune response plays an important role in the production of lesions in the host. Such lesions are characteristic of the disease during infection with certain viruses. For instance they occur for infections with hepatitis B virus (Ando et al., 1993), LCMV (Buchmeier et al., 1980; Bosma et al., 1983; Custer et al., 1985), Sindbis virus (Levine et al., 1991; Levine and Griffin, 1993) and Semliki Forest virus (Levine et al., 1991; Fazakerley and Buchmeier, 1993). These viruses, normally pathogenic in immunocompetent mice, are avirulent or produce reduced pathology in animals that are unable to mount an effective immune response.

There are currently a few reports where B- and T-cell-deficient mice with a defect in the recombinase-activation gene function-1 (RAG-1-/-) have been used in the study of encephalitic falaviviruses (Mombaerts et al., 1992; Diamond et al., 2003; Hebeis et al., 2004), and there are some reports where mice with the severe combined immune deficiency mutation (SCID) (Bosma et al., 1983; Halevy et al., 1994; Campbell and Pletnev, 2000; Pletnev et al., 2000; Leyssen et al., 2001; Pletnev, 2001; Charlier et al., 2002; Leyssen et al., 2003) have been used. The use of these immunodeficient mice has helped to understand the pathogenesis of WNV (Halevy et al., 1994; Diamond et al., 2003). However, some aspects of the WNV pathogenesis differ from MVE/JEV pathogenesis, as discussed in section 1.4 of this thesis. Thus, the use of RAG-1-/- mice for the study of MVE pathogenesis is warranted.

Studies of flaviviruses in SCID mice are useful to elucidate some of the mechanisms of flavivirus neuroinvasiveness (Halevy et al., 1994; Chambers and Nickells, 2001), tissue tropism (Mathur et al., 1981; Wu et al., 1995; An et al., 1999; Chambers and Nickells, 2001) and for therapeutic purposes against flavivirus infections (Leyssen et al., 2001; Charlier et al., 2002). Nude mice that lack functional T cells (Nakamura et al., 1985) and immunocompetent mice have been used for infection with the encephalitic flavivirus JEV, but disease only occurred in the former (Lad et al., 1993). The interpretation of these results remains ambiguous. T-cell help is required to switch B-cell immunoglobulin synthesis to the IgG isotype, which has a virus-neutralizing property (Uren, 1987; Mathews et al., 1991; Mathews et al., 1992). Thus, the lack of protection of nude mice from JEV challenge could be due to the lack of a direct protective T-cell response, or to the lack of T help for the proper Ab response. Clear cases of flavivirus-induced T-cell immunopathology exist (Hirsch and Murphy, 1968; Camenga and Nathanson, 1975; Semenov, 1975; Semenov et al., 1975); however,

there are many contradicting studies and the role of the cellular immune response in the protection or in the enhancement of flavivirus pathogenesis is unclear (for reviews see (Monath, 1986; Hill, 1993; Mullbacher et al., 2003)).

An additional *in vivo* approach, which addresses the specific role of $CD8^+$ T cells during flavivirus infections, is the use of mice with a knockout mutation in the ß2-microglobulin (ß2-M-/-) gene. The ß2-M-/- mice lack cell-surface expression of MHC class I antigen, which is necessary during positive selection of $CD8^+$ T cells, and consequently these mice are deficient in $CD8^+$ T cells (Koller et al., 1990; Zijlstra et al., 1990). Thus, the mouse model allows assessment of the role of $CD8^+$ T lymphocytes in viral infection.

In this chapter, I use RAG-1-/- and ß2-M-/- mice to investigate if MVE-induced CNS disease is the result of viral infection per se, and/or if it is due to pathology mediated by lymphocytes. I also discuss complementary studies on MVE-immune T- and B-cells following passive transfer to MVE-infected wt mice, and investigate what their role is in the recovery from an infection, or if they contribute to disease.

5.3 Results

5.3.1 Mortality of RAG-1-/-, ß2-M-/- and wt mice after infection with 10^2 and 10^8 PFU of MVE, iv

I injected RAG-1-/-, ß2-M-/- and wt mice iv with an MVE dose of 10^2 PFU, to compare their susceptibility to the infection. Mortality in RAG-1-/- mice was significantly higher (91.3% versus 47% in the wt controls; $P = 0.003$), but delayed (17.1 versus 11.5 days pi in the wt controls; $P = 0.04$) in comparison with wt mice (Table 5.1). Contrary to the increase in mortality of RAG-1-/- mice, the mortality in ß2-M-/-

mice was significantly reduced (13.7%: $P = 0.03$), and the ATD (13.7 days pi) delayed in comparison to wt mice (Table 5.1). The latter suggests that Tc cells played a detrimental role in wt mice infected with MVE.

Infection of ß2-M-/- mice with 10^8 PFU of MVE, iv, resulted in 100% mortality. However, the ATD was delayed by ~1 day relative to wt mice (Table 5.1). I also infected three RAG-1-/- and three wt mice of 11 weeks of age with 10^8 PFU of virus iv (data not shown). One of the RAG-1-/- mice died at day 7 pi, while the other two died at day 11 pi. Two of the wt mice died at day 6 and one survived. Although there were only very small groups of 11-week-old mice available, the experiment revealed that there was a similar trend in the development of the disease as in the 6-week-old mice: RAG-1-/- mice died in higher numbers but with a delayed time to death.

Table 5.1 Mortality of 6-week-old C57Bl/6 mice after infection with 10^2 or 10^8 PFU of MVE, iv[a]

Dose (PFU)	Mouse strain	N[b]	% mortality	ATD ± SEM[c]
10^2	wt	17	47.0	11.5 ± 0.6
	RAG-1-/-	23	91.3	17.1 ± 2.2
	ß2-M-/-	22	13.7	13.7 ± 1.2
10^8	wt	19	100.0	5.5 ± 0.2
	ß2-M-/-	9	100.0	6.7 ± 0.4

[a] Mortality ratios are given for wt and mutant mice.
[b] Number of infected mice per group.
[c] Average time to death (ATD), calculated in days ± standard error of the mean (±SEM).

5.3.2 Kinetics of MVE growth in extraneural organs and brains of RAG-1-/- and ß2-M-/- mice

To assess the role of B and T cells in the clearance of MVE, I used 6-week-old mice that are deficient in both effector cells (RAG-1-/-), and mice that lack effector Tc cells (ß2-M-/-); I used immunocompetent wt mice as controls. All mice received doses of 10^2 or 10^8 PFU of virus iv to compare the kinetics of virus growth in organs such as spleen, lymph nodes (lymph nodes were not collected from RAG-1-/- mice due to their minute size), muscle and brain. The virus titers for wt mice infected with 10^8 PFU were taken from chapter 4. I collected extraneural tissues of the mice infected with 10^2 PFU at days 6 and 8, brains at days 6, 8,12 and 14, and tissues of the mice infected with 10^8 PFU at days 2, 3 and 4 pi. To test for viremia, I collected sera samples at days 1 and 2 after infection with 10^2 PFU of virus iv.

Virus growth in extraneural organs

I expected that the chance to detect virus in RAG-1-/- mice would be higher than in wt mice because the former would lack T- and B-cells. However, virus titers were below the limit of detection (10^3 PFU/g for spleen and muscle and 10^2 PFU/ml for serum; data not shown) after infection with 10^2 PFU. For ß2-M-/- and wt mice, spleen, muscle and lymph nodes were tested in parallel. Results of virus load in ß2-M-/- mice were also below the detection limit (data not shown). I also compared virus distribution in spleen and muscle of two moribund RAG-1-/- and wt mice infected with 10^2 PFU iv of MVE and sacrificed on days 12 and 14 pi. The spleen virus titers in RAG-1-/- and wt mice at day 14 pi were both low (~ 10^3 PFU/g). The virus content in muscle of a

RAG-1-/- mouse at day 12 pi was slightly higher (1.5 x 10^4 PFU/g) than the 4 x 10^3 PFU/g found in the wt mouse (data not shown).

When mice were infected iv with 10^8 PFU, virus titers in spleen of wt and ß$_2$-M-/- mice marginally decreased between days 2 and 4 pi. The opposite occurred in spleen of RAG-1-/- mice, where virus titers started to appear a day later than in the other mouse strains and were all positive on day 4 pi. Most of the spleens from wt and ß$_2$-M-/- mice showed no detectable titers by that day (Fig. 5.1 A).

The results from virus titration in lymph nodes after infection with 10^8 PFU, iv, are shown in Fig. 5.1 B. Lymph nodes of ß$_2$-M-/- mice had comparable titers to those of wt mice, and showed a similar decline between days 2 and 4 pi.

Of the muscle tissues from mice that were infected with 10^8 PFU, iv, only samples from wt and RAG-1-/- mice had increased titers from day 2 to day 4 pi, while a muscle sample from a ß$_2$-M-/- mouse showed marginal virus titers of 10^3 PFU/g only at day 2 pi (Fig. 5.1 C).

In general, spleen and muscle from RAG-1-/- mice after infection with 10^8 PFU appear to support slightly higher (although delayed in the spleen) virus replication than tissues from ß$_2$-M-/- and wt mice. Results obtained from the infection of wt and ß$_2$-M-/- mice were comparable. They indicate that $CD8^+$ lymphocytes do not appear to reduce virus growth.

Kinetics of neuroinvasion and growth of MVE in brains of infected RAG-1-/- and ß2-M-/- mice

Groups of RAG-1-/-, ß$_2$-M-/- and wt mice, infected with a dose of 10^2 PFU of MVE, iv, showed no detectable virus titers in the brain at days 6 and 8 pi (Fig. 5.2 A;

only small groups of mice were available for this experiment). When I collected brain tissues from moribund RAG-1-/- mice at later times (days 12 and 14 pi), I not only observed positive titers, but also titer values of at least two logs higher than for the wt mice that were injected in parallel (Fig. 5.2 A). At day 12 pi, brain virus titers were as high as 6.2×10^9 PFU/g in the moribund RAG-1-/- mouse, compared with 1.6×10^7 PFU/g in the moribund wt mouse. At day 14 pi, MVE titers in brain tissue were 4.4×10^9 PFU/g in the RAG-1-/- mouse, versus 1.3×10^6 PFU/g in the wt mouse. I also tested the brains of two ß$_2$-M-/- asymptomatic mice that were infected at the same time as the wt and RAG-1-/- mice with the same dose of 10^2 PFU of MVE iv, at days 12 and 14 pi. These tests were negative (Fig. 5.2 A), which is consistent with the significant reduction in mortality that occurred in wt and RAG-1-/- mice (Table 5.1).

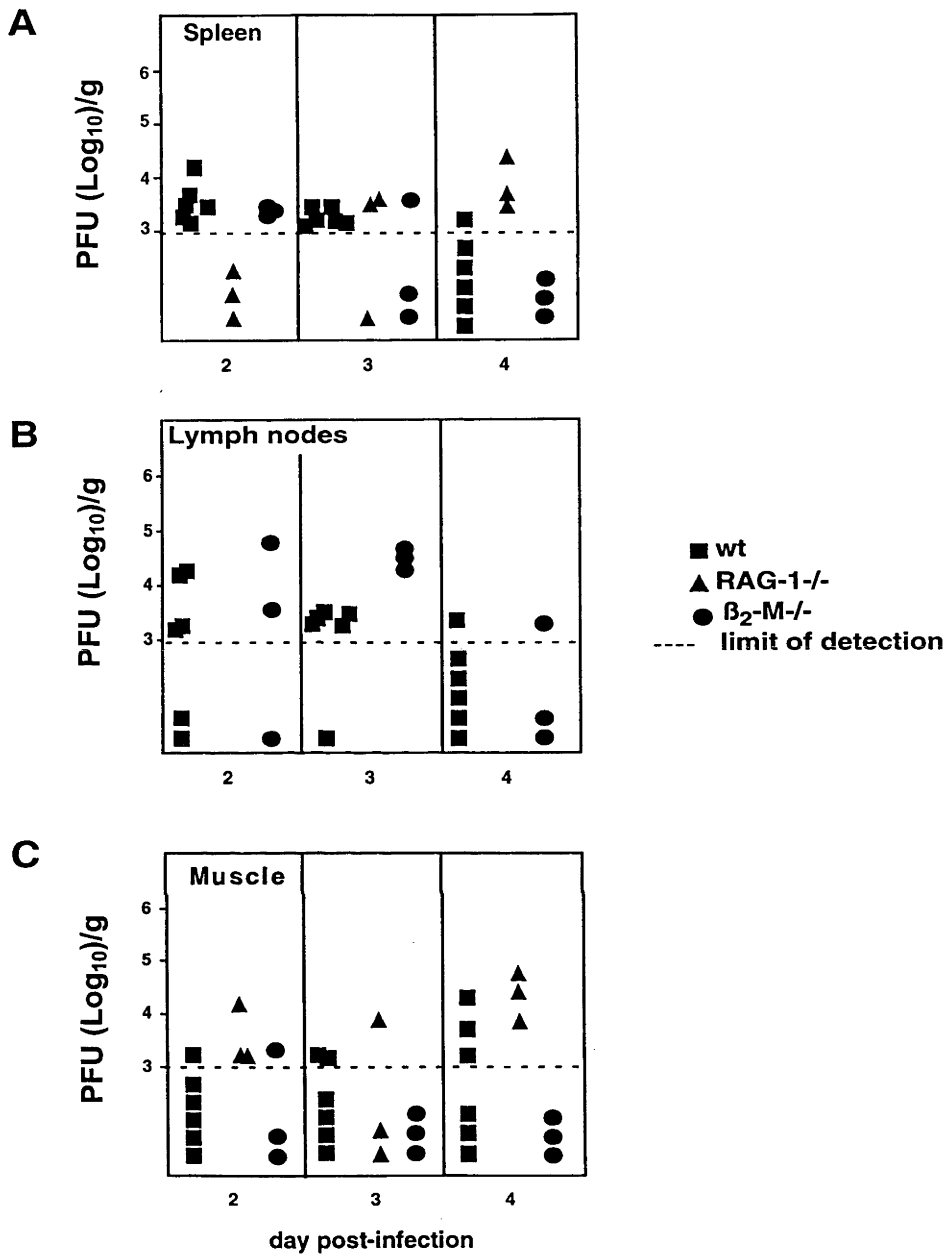

Fig. 5.1 Growth of MVE in extraneural tissues of RAG-1-/- (▲), ß2-M-/- (●) and wt mice (■). Six-week-old mice were infected with 10^8 PFU of MVE iv. At the indicated times, animals were sacrificed and virus titers in the spleen (**A**), lymph nodes (**B**) and muscle (**C**) were determined by plaque assay. The limit of detection of virus titers was 1 x 10^3 PFU/g of wet tissue and is indicated by the interrupted line.

Figure 5.2 B shows a comparison of virus titers in brain tissue from RAG-1-/- and ß2-M-/- mice, relative to those from wt mice infected with 10^8 PFU of MVE. Virus titers increased in all the mouse strains between days 2 and 4 pi. At day 2 pi, virus content in the brains of wt and ß2-M-/- mice was in the range of $<10^3$ to $>10^6$ PFU/g, but did not exceed 10^4 PFU/g for the RAG-1-/- mice. At day 3 pi, one out of six wt brains tested negative, but all the RAG-1-/- and ß2-M-/- brains showed positive titers. The lowest virus titers in the brain were in the range of 10^3 PFU/g in wt mice, 10^5 PFU/g in RAG-1-/- mice, and 10^6 PFU/g in ß2-M-/- mice, but all three strains had comparable maximum titers of $2x10^8$ PFU/g. All brains from the RAG-1-/- and ß2-M-/- mice at day 4 pi had virus titers in the range of 10^9 PFU/g. In contrast, three out of six wt mice showed a virus content in brain that was 4 logs lower. It is of interest that, despite 100% mortality found in the groups of 6-week-old wt and ß2-M-/- mice that were inoculated with 10^8 PFU of MVE, iv, (mortality of RAG-1-/- mice was not tested at this dose), a significant number of wt — but not ß2-M-/- mice — had low or undetectable titers in the brain on days 3 and 4 pi.

5.3.3 Anti-MVE Ab response in ß2-M-/- mice

Because ß2-M-/- mice still retain their $CD4^+$ T-cell function, T-cell help is available for induction and isotype switching in anti-viral humoral immunity. I compared antibody levels in ß2-M-/- and wt mice in parallel, after they were infected with 10^5 PFU of virus, iv. Serum samples were tested at days 5, 10, 15 and 21 pi. Mice of both strains showed 100% seroconversion at all time points. Ab titers ranged from 2.0 to 4.1 Log_{10}, with no marked difference between groups of ß2-M-/- and wt mice (Table 5.2). I also confirmed that sera from MVE-infected RAG-1-/- mice did not show virus-reactive Ab detectable by ELISA.

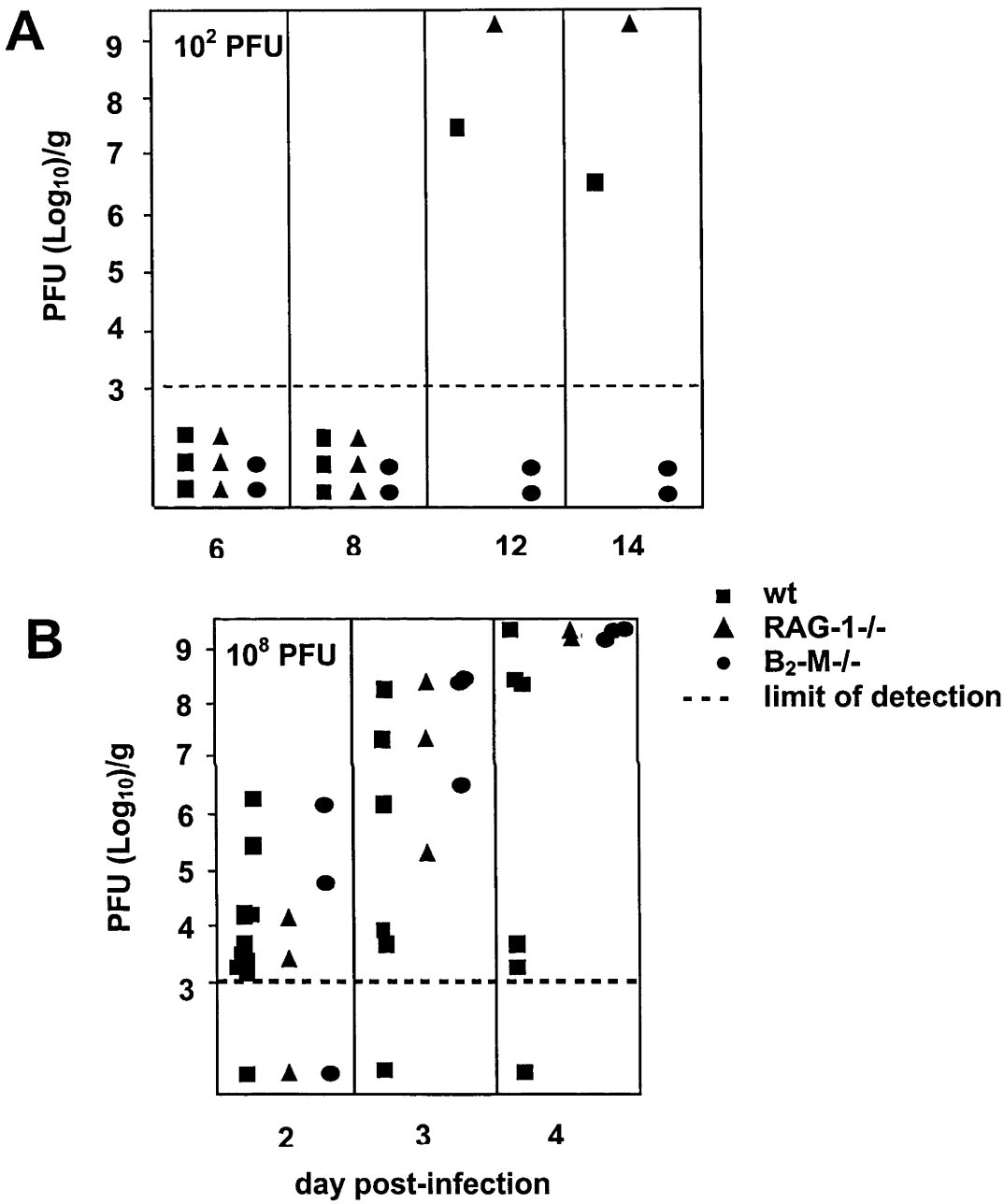

Fig. 5.2 Growth of MVE in brains of RAG-1-/- (▲), ß2-M-/- (●) and wt mice (■). Six-week-old mice were infected with 10^2 (**A**) and 10^8 (**B**) PFU of MVE iv. At the indicated times, animals were sacrificed and virus titers were determined by plaque assay. Control wt mice were not infected in parallel with the immunodeficient mice for the dose of 10^8 PFU. The lower limit of detection of virus titers was 1 x 10^3 PFU/g of wet tissue and is indicated by the interrupted line.

Table 5.2 MVE-specific Ab production in 6-week-old C57Bl/6 wt and in ß$_2$-M-/- knockout mice after infection with 10^5 PFU, iv

Mouse strain	No./ group	Day pi	% Seroconversion	Mean Ab titer, log_{10} (range)[a]
wt	3	5	100	2.3 (2.0-2.6)
ß$_2$-M-/-	3	5	100	2.2 (2.0-2.6)
wt	3	10	100	3.4 (3.2-3.5)
ß$_2$-M-/-	3	10	100	3.5 (3.2-3.8)
wt	3	15	100	3.6 (3.5-3.8)
ß$_2$-M-/-	3	15	100	3.7 (3.5-3.8)
wt	3	21	100	3.8 (3.5-4.1)
ß$_2$-M-/-	3	21	100	4.0 (3.8-4.1)

[a] MVE-specific antibody titers were determined by ELISA, and end points were calculated as described in Materials and Methods, Chapter 2.

5.3.4 Histopathology in brain and spleen of RAG-1-/- and ß2-M-/- mice

Table 5.3 shows a summary of histopathological manifestations and virus load for corresponding spleen and brain samples of RAG-1-/-, ß2-M-/- and wt mice infected with 10^8 PFU of MVE, iv. The data from wt mice are taken from Table 4.3 for comparison. None of the samples from the two immunodeficient mouse strains showed histopathology (except one brain sample of a RAG-1-/- mouse at day 4 pi), despite frequently showing high virus titers. This result is distinct from that for wt mice, which generally had a histopathology that corresponded with the presence of virus in brain on day 4 pi.

Table 5.3 Histopathology results of ß2-M-/-, RAG-1-/- and control wt mice infected with 10^8 PFU of MVE iv

		Brain		Spleen	
Mouse strain	**Day pi**	**Histology**	**Plaque assay**	**Histology**	**Plaque assay**
ß2-M-/-	2	normal	$1.5x10^6$	normal	$4x10^3$
	2	normal	$8x10^4$	normal	$1.5x10^3$
	2	normal	$<10^3$	normal	$2x10^3$
	3	normal	$2.7x10^8$	normal	$<10^3$
	3	normal	$3.5x10^6$	normal	$4x10^3$
	3	normal	$1.3x10^8$	normal	$<10^3$
	4	normal	$2.7x10^9$	normal	$<10^3$
	4	normal	$4.3x10^9$	normal	$<10^3$
	4	normal	$7.2x10^9$	normal	$<10^3$
RAG-1-/-	2	normal	$1x10^4$	normal	$<10^3$
	2	normal	$1.5x10^3$	normal	$<10^3$
	2	normal	$<10^3$	normal	$<10^3$
	3	normal	$1.8x10^5$	normal	$<10^3$
	3	normal	$4.5x10^8$	normal	$4x10^3$
	3	normal	$3.6x10^7$	normal	$4x10^3$
	4	normal	$1x10^9$	normal	$6x10^3$
	4	extensive pvi	$9x10^9$	normal	$3x10^3$
	4	normal	$4.9x10^9$	normal	$1.7x10^4$
wt	2	normal	$1.5x10^4$	reactive, red pulp enlarged, white pulp with secondary follicles	$5x10^3$
	2	normal	$2x10^4$	reactive, red pulp enlarged, white pulp with secondary follicles	$2x10^3$
	2	normal	$4.5x10^3$	red pulp enlarged	$5x10^3$
	3	normal	$2.2x10^6$	reactive	$1.5x10^3$
	3	normal	$9.5x10^3$	reactive	$4x10^3$
	3	extensive pvi	$2.2x10^7$	reactive	$3x10^3$
	4	extensive pvi	$2.7x10^9$	reactive	$1.5x10^3$
	4	extensive pvi	$6x10^8$	reactive	$<10^3$
	4	mainly pvi	$5.4x10^8$	reactive	$<10^3$

normal: no histological lesions, similar to mock-infected mice (data not shown).
pvi: perivascular infiltration.
reactive: reaction of the spleen to antigens with the development of secondary follicles.

5.3.5 Passive transfer of MVE-primed B and T cells to MVE-infected wt mice

The results in this chapter show, somewhat paradoxically, that mice that lack effector Tc cells (β_2-M-/- mutation) are significantly more resistant to MVE infection than wt mice. Thus, it was of interest to know if passive transfer of anti-MVE B or T cells to wt recipient mice would give rise to immunopathology.

I generated anti-MVE effector cells by infecting wt donor mice with10^2 PFU of MVE, iv, and collected spleens 6 days pi. I separated $CD4^+$ and $CD8^+$ T cells from B cells with nylon wool and injected 4 x 10^7 anti-MVE effector B or T cells into wt mice that had three days previously received 10^2 PFU of MVE, iv. The controls were six mock-infected mice that received anti-MVE T or B cells, and six mice that were iv infected with 10^2 PFU of MVE only. The possibility of virus contamination in transferred splenocytes was tested by plaque assay: the assays were all negative. The fact that mock-infected recipient mice did not show signs of infection also suggests that no infective virus was contaminating the transferred cells.

All five mice that received MVE-immune B cells (78% pure) survived MVE infection, whilst 50% died in the control group of six mice that received the virus but not the immune B cells (Fig. 5.3 A).

Passive transfer of MVE-primed T splenocytes (73% pure) into MVE-infected mice resulted in 66% mortality in a group of six MVE-infected recipients. Of the seven infected non-recipients, 42.8% died, and none of the mock-infected recipients died (Fig. 5.3 B). It is not known if contaminating B cells among transferred T splenocytes contributed to survival. Thus, due to difficulties in obtaining pure B- and T-cell populations, the transfer experiments have to be interpreted with some reservation. Nevertheless, they show interesting trends, particularly in the T-cell transfers. In

summary, passive transfer of MVE-immune B cells offers protection, while transfer of T cells to MVE-infected wt mice does not offer protection to the infected recipients, but shows a slight (although statistically not significant) increase in mortality compared with the non-recipient infected mice.

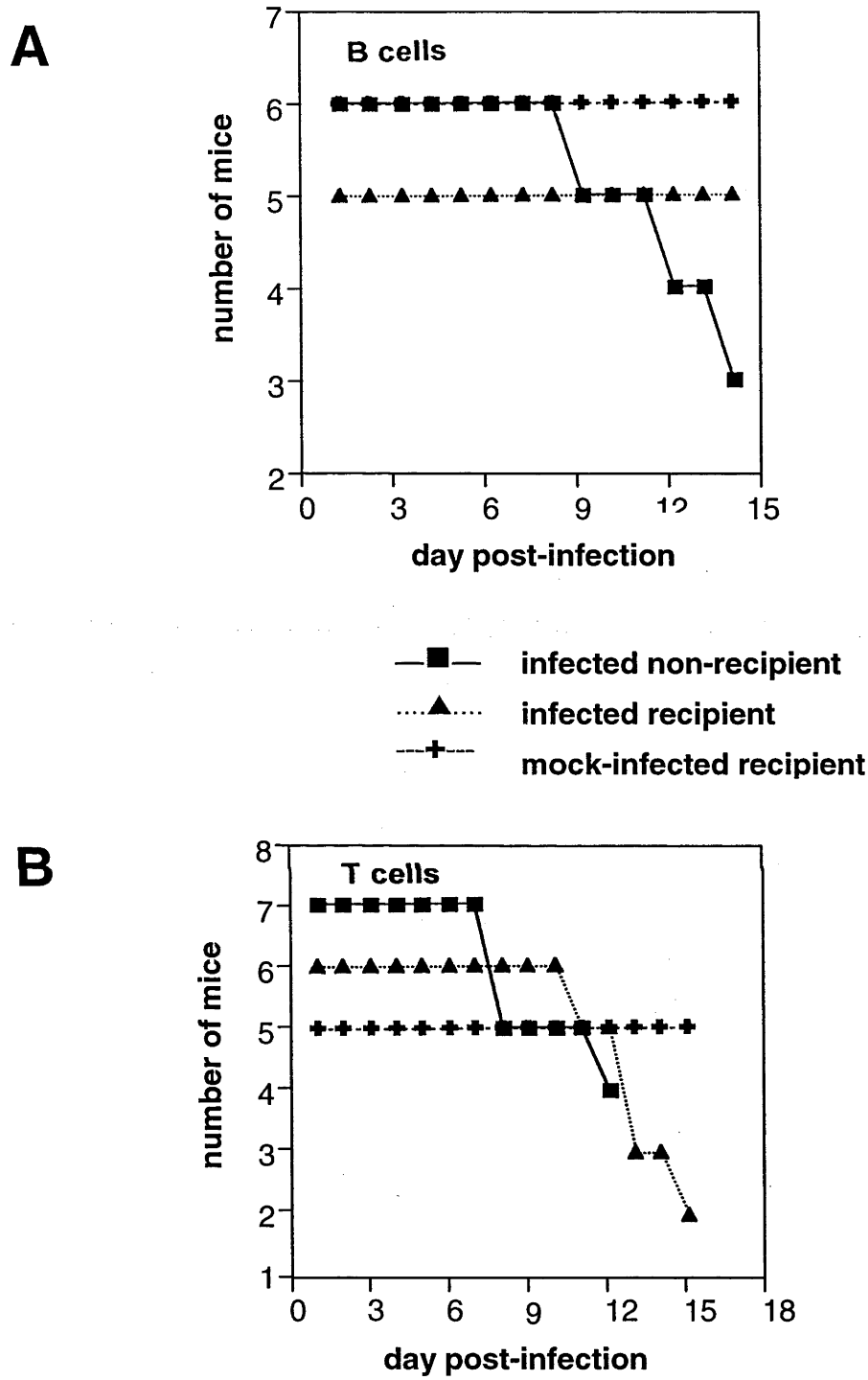

Fig. 5.3 Mortality in groups of wt mice infected iv with 10^2 PFU of MVE, after passive transfer of MVE-immune B cells (**A**) and MVE-immune T cells (**B**).

5.4 Discussion

Due to their B- and T-cell deficiencies, respectively, RAG-1-/- and $ß_2$-M-/- animals provide unique mouse models to assess the role of the two lymphocyte populations in recovery from, or exacerbation of, MVE infection. To complement the two mouse models, passive transfer of MVE-immune B and T cells to MVE-infected wt recipients was performed.

Mortality of RAG-1-/- mice after infection with a low MVE dose was significantly increased ($P = 0.003$), but accompanied by a delay in ATD of 6 days ($P = 0.04$) compared to wt mice. The high mortality of MVE-infected RAG-1-/- mice was most likely due to the lack of an Ab response. This lack probably occurred because there was protection provided by transfer of MVE-immune B cells. Such a lack was also shown in numerous studies by others that studied the importance of Ab in survival of flavivirus infection (Mathur et al., 1982; Heinz et al., 1983; Mathews and Roehrig, 1984; Monath, 1986; Schlesinger et al., 1986; Kimura-Kuroda and Yasui, 1988; Kaufman et al., 1989; Mason et al., 1989; Schlesinger and Chapman, 1995; Kreil and Eibl, 1997; Broom et al., 2000; Roehrig et al., 2001; Ben-Nathan et al., 2003; Diamond et al., 2003). The small number of RAG-1-/- mice that survived infection with a low MVE dose in my experiments could be due to protection conferred by their unimpaired innate immunity. The fact that the immunodeficiency in RAG-1-/- mice could prolong survival, but at the cost of increased mortality, strongly indicates that the T-cell immune response may have a pathogenic role in MVE infection. Consistent with the delayed ATD in rag mice infected with a low dose of MVE, clinical signs of disease and virus in the brain were detectable ~4 days later than in wt mice. However, virus content in the brain of RAG-1-/- mice at late times pi was very high ($>10^9$ PFU/g). This probably reflects that there was a reduced virus clearance in the brain due to the loss of B- and T-

cell function, and/or that the longer time interval allowed virus to grow in the brain prior to the death of the animals.

Results on MVE load in tissues of RAG-1-/- mice after infection with 10^8 PFU showed that these mice had a delayed, but more efficient, virus replication than ß2-M-/- and wt mice. At the early time pi, virus load in the brains of RAG-1-/- mice was lower than virus load in the brains of wt and ß2-M-/- mice. However, at later times, virus titers in brains of RAG-1-/- mice were as high as for wt and ß2-M-/- mice. The delayed but increased mortality and detection of virus in RAG-1-/- mice suggests both a negative and positive input of the adaptive immune response: negative, in the sense that lack of adaptive immunity seems to delay virus entry into the brain and mortality; and positive, in the sense that the immune responses are required to prevent massive virus replication and increased mortality.

Contrary to the results obtained in RAG-1-/- mice, the mortality of ß2-M-/- mice after infection with a low dose of MVE iv, was significantly reduced ($P = 0.03$) relative to wt mice. This suggests that $CD8^+$ T cells are detrimental in recovery from MVE infection in mice. Alternatively, cell surface MHC class I could have played a role in MVE infection, for instance as a receptor for the virus. However, this latter notion was refuted by the experiments discussed in Chapters 6 and 7. A more efficient humoral immune response is unlikely to have contributed to the increased resistance of ß2-M-/- mice to MVE (relative to wt mice). This result is similar to results found with other virus models such as vaccinia and vascular stomatitis (Spriggs et al., 1992). Virus-specific Ab titers in serum of MVE-infected ß2-M-/- and wt mice did not differ significantly. Chapters 6 and 7 further explore and discuss a putative $CD8^+$ T cell-mediated mechanism for virus entry into the brain that may have accounted for the

increased survival of ß$_2$-M-/- mice from infection with MVE in the absence of CD8T$^+$ cells.

In the absence of CD8$^+$ T cells, growth of MVE in extraneural tissues was not significantly augmented relative to wt mice. Accordingly, the poor growth of MVE in extraneural tissues of adult mice, in general, is not the result of an anti-viral function of MVE-immune CD8$^+$ T cells but more likely due to innate immunity, in particular type I interferon, as shown in Lobigs et al. (2003). It is possibly an early antiviral Ab response. The fact that MVE-infected ß$_2$-M-/- mice did not show more virus replication in the brain than wt mice has been observed in other virus models. For example, infection of ß$_2$-M-/- mice with Sindbis virus (SV) (Kimura and Griffin, 2000) or reovirus (Major and Cuff, 1997) resulted in virus clearance. In the case of SV, there was no difference in virus load compared with to wt mice (Kimura and Griffin, 2000). It has been postulated that CD4$^+$ T cells may play a more prominent role in control of virus infection when CD8$^+$ T cells are absent, by increasing their cytotoxicity and numbers. For example, phenotypic analysis of splenic lymphoid cells of ß$_2$-M-/- mice showed that there was an increase in CD4$^+$ cell subsets (Spriggs et al., 1992).

Histological analyses of brains from MVE-infected ß$_2$-M-/- and RAG-1-/- mice show that there is less inflammatory cell infiltration than in the brains of infected wt mice. This is consistent with their respective lack of splenocyte subpopulation, and with studies that show that most of the inflammatory cells in the brains of 6-week-old WNV-infected wt mice are CD8$^+$ T cells (Liu et al., 1989; Wang et al., 2003). Wang et al. also showed that ß$_2$-M-/- mice infected with WNV had reduced histopathology and a prolonged survival time (Wang et al., 2003).

Experiments on the passive transfer of MVE-immune T or B cells into MVE-infected wt mice give some indication on the role of these cells in the recovery from, or

exacerbation of, infection. Transfer of T cells to MVE-infected wt mice does not contribute to protection; on the contrary, it appears to give rise to a slight increase in mortality. Given that no death occurred in any of the recipient mice, passively transferred MVE-immune B cells clearly have a protective function,.

In summary, the results in this chapter indicate that B cells are vitally important for protection from MVE infection, a finding which is consistent with previous studies (Mathur et al., 1982; Heinz et al., 1983; Mathews and Roehrig, 1984; Monath, 1986; Schlesinger et al., 1986; Kimura-Kuroda and Yasui, 1988; Kaufman et al., 1989; Mason et al., 1989; Roehrig et al., 1989; Schlesinger and Chapman, 1995; Kreil and Eibl, 1997; Broom et al., 2000; Libraty et al., 2002; Ben-Nathan et al., 2003; Diamond et al., 2003). Contrarily, the lack of $CD8^+$ T cells has a positive effect on the recovery from MVE infection. The main mechanisms of cytotoxicity of T cells, namely the degranulation and Fas pathways, could be responsible for the increased pathology mediated by $CD8^+$ T cells in MVE infection, and are studied in Chapters 6 and 7.

Chapter 6

Degranulation- and Fas-mediated cytotoxicity play no protective role in the pathogenesis of MVE-infected mice

6.1 Summary

In this chapter, I describe MVE pathogenesis in mice with a deficiency in different subsets of the effector functions of cytotoxic (NK/Tc) cells. I used C57Bl/6 mice with knockout (ko) mutations in the perforin gene (perf-/-), the granzymes A and B genes (gzmAxB-/-), and the perfxgzmAxB-/- genes to evaluate the role of the degranulation pathway of cytotoxicity during MVE challenge. Fas ligand mutant (*gld*) mice, and Fas receptor mutant (*lpr*) mice allowed the investigation of the role of the Fas pathway of cytotoxicity in recovery or disease following MVE infection. Mice with a deficiency in the gamma interferon receptor (IFN-γR-/-) were also tested. I used syngeneic wild-type (wt) mice as controls. Most of the immunodeficient mice (except the *lpr* and IFN-γR-/- mice) showed a reduction in mortality and morbidity, compared with the wt controls. However, the virus load in tissues of mice with a deficiency in cell-contact-mediated cytotoxicity (perforin/granzymes and Fas) was slightly increased, relative to that of controls. Additionally, these mice showed earlier neuroinvasion of the virus, in comparison with wt mice. These data confirm the importance of cell-mediated cytotoxicity in virus clearance, but also suggest that it can contribute to increased disease.

6.2 Introduction

The humoral immune response is the main mechanism of protection during flaviviral infections (Heinz et al., 1983; Mathews and Roehrig, 1984; Schlesinger et al., 1986; Kimura-Kuroda and Yasui, 1988; Kaufman et al., 1989; Mason et al., 1989; Schlesinger and Chapman, 1995; Kreil and Eibl, 1997; Broom et al., 2000; Roehrig et

al., 2001; Libraty et al., 2002; Ben-Nathan et al., 2003; Diamond et al., 2003a; Diamond et al., 2003b). However, humoral immunity in primary flavivirus infections is not always sufficient to prevent fatal encephalitis.

Cytotoxic NK and Tc cells exert their effector function by two different mechanisms: 1) by cellular cytotoxicity, through the degranulation pathway (perforin/granzymes) or the Fas pathway (Kagi et al., 1994) and 2) by cytokines such as gamma interferon (IFN-γ) (Boehm et al., 1997) and tumor necrosis factor alpha (TNF-α) (Vassalli, 1992). The cytotoxic functions of NK and Tc cells are critical for immunity against some virus infections, and also contribute to the pathogenesis of other viral diseases (Harty et al., 2000). IFN-γ is crucial to control a number of neurotropic viral infections (Finke et al., 1995; Geiger et al., 1997). The importance of IFNs in the recovery from encephalitic flavivirus infections has been shown *in vivo* through the administration of IFN inducers (Haahr, 1971; Vargin et al., 1977; Taylor et al., 1980) and through the administration of recombinant IFNs (Pinto et al., 1988; Brooks and Phillpotts, 1999). The presence of IFN-γ *in vitro* studies has shown inhibition of JE virus production by infected monocytes (Hasegawa et al., 1990).

Mice used in this study — perf-/- single ko mice, gzmAxB-/- double ko mice, and perfxgzmAxB-/- triple ko mice — are viable, fertile and display normal numbers of $CD8^+$ and NK cells (Blink et al., 1999), in which the Fas pathway of cytotoxicity is still functional. On the other hand, a deficiency in either Fas (*lpr* mutation) or Fas ligand (*gld* mutation) results in accumulation of aberrant T cells, which leads to lymphoadenopathy and splenomegaly (Igney et al., 2000). Thus, Fas-deficient mice die of lymphoproliferative disease around 25 weeks of age (Kagi et al., 1999). Mice with a targeted disruption of the IFN-γR-/- gene help to investigate the role of that cytokine (made exclusively by NK and T cells) during MVE pathogenesis. The IFN-γR-/- mice

have an impaired production of nitric oxide by monocytes and a reduced expression of MHC classes I (Chou et al., 2000) and II (Dalton et al., 1993). The IFN-γR-/- mice have been used during infection with dengue (Johnson and Roehrig, 1999) and yellow fever (Liu and Chambers, 2001) viruses in mice. The IFN-γR-/- mice have been recently used in the infection of MVE by Lobigs et al. (Lobigs et al., 2003).

The specific aims of the work described in this chapter were therefore: a) to characterize the role of perforin, granzymes A and B, Fas and IFN-γ in MVE pathogenesis and recovery, by using mice with a deficiency in those effector molecules, and b) to assess the role of cytotoxicity versus other effector mechanisms of antiviral immunity (e.g. the antibody response) during *in vivo* MVE infection.

6.3 Results

6.3.1 Mortality caused by MVE in mice with a deficiency in degranulation, Fas, and IFN-γ receptor

In the experiments described in this chapter, I infected 6-week-old perf-/-, gzmAxB-/-, perfxgzmAxB-/-, *gld* and *lpr* mice with doses ranging from 0.1 to 10^8 PFU of MVE iv, and one group of IFN-γR-/- mice of matching age with 10^2 PFU of MVE iv. Subsequently, I observed the mice for three weeks after infection, and recorded the percentage mortality and average time to death (ATD) (Table 6.1).

Role of the degranulation pathway (perforin and granzymes) of cytotoxicity in MVE pathogenesis

The groups of perf-/-, gzmAxB-/-, and perfxgzmAxB-/- ko mice that were inoculated with virus doses of 0.1, 10^2, 10^3 and 10^5 had reduced mortality in comparison to wt mice. There was no dose-dependent increase in mortality. All ko mice infected with 0.1 PFU of virus showed a slightly reduced and delayed mortality rate, when compared with wt mice. Mortality in perf-/- mice was 33% ($P = 0.18$), in gzmAxB-/- mice 28% ($P = 0.07$) and in perfxgzmAxB-/- mice 33% ($P = 0.37$), in contrast to 59% mortality in wt mice (Table 6.1). After infection with 10^2 PFU of MVE, mice showed the following percentages of mortality: perf-/- mice 20% ($P = 0.09$), gzmAxB-/- mice 25% ($P = 0.12$), perfxgzmAxB-/- mice 32% ($P = 0.86$), and wt mice (46%). Of the ko mice, I only infected a group of perf-/- mice with a dose of 10^3 PFU of MVE. These, consistent with the 0.1 and 10^2 PFU doses, produced a reduced mortality of 38% ($P = 0.43$), in comparison with a mortality of 56% in wt mice. The mortality after infection with a dose of 10^5 PFU of MVE was 33% ($P = 0.09$) in perf-/- mice, compared with 40% in the wt mice. When the mortality data, obtained from the doses that gave rise to close to 50% mortality (0.1 to 10^5 PFU) for the perf-/- and gzmAxB-/- mice are summed up, a statistically significant increase in resistance to MVE infection is found for the two strains ($P = 0.022$ and 0.014, respectively; Fisher's exact test). Given the relatively small number of perfxgzmAxB-/- mice that were available for this study, the difference in combined mortality between these ko mice (8 dead, 17 alive) and the wt mice (59 dead, 59 alive) is not statistically significant ($P = 0.125$).

All mouse strains infected with 10^8 PFU of virus showed 100% mortality after 5 to 6 days pi. However, the ATD was marginally delayed, in comparison with the wt mice, in mice that lacked subsets of the cytotoxic pathways (Table 6.1). On average, wt

mice died at day 5.5, perf-/- mice at day 6.6, gzmAxB-/- mice at day 6.4, and perfxgzmAxB-/- mice at day 9.4 pi, showing a significant increase in ATD relative to wt mice ($P = 0.009$; Mann-Whitney test). Clinical signs appeared a day earlier in the wt mice (day 4) than in most of the mutant mice (data not shown). Additionally, the majority of the mutant mice showed clinical disease (i.e., encephalitis and hind leg paralysis) for a shorter period (2–3 days) than the wt mice, which developed severe clinical signs of disease early, and died within 24 h of disease onset (data not shown).

In summary, the data in Table 6.1 indicate that the granule exocytosis-mediated cytotoxicity of NK/Tc cells has no protective value in MVE infection, and that it appears to accelerate the fatal outcome of viral growth in the CNS, and increase the mortality rate.

Role of the Fas-mediated pathway of cytotoxicity in MVE pathogenesis

When I infected groups of *lpr* and *gld* mice with 0.1 PFU of MVE, they had a mortality of 40% and 33% respectively; this is a reduced mortality compared with wt mice (59%), although not statistically significant, with *P*-values of 0.46 for *lpr* mice and 0.37 for *gld* mice in comparison with wt mice (Table 6.1). After MVE infection with 10^2 PFU, *gld* mice showed a slightly reduced mortality, of 27% ($P = 0.33$), compared with 46% in the wt mice, but *lpr* mice showed a slightly increased mortality of 55%, an increase that was not statistically significant ($P = 0.74$). A viral dose of 10^5 PFU resulted in a similar situation: a slightly reduced mortality for the *gld* mice (36%; $P = 0.69$) and an increased mortality in the *lpr* mice (80%; $P = 0.20$) in comparison with the wt mice (40%). The mortality after infection with 10^8 PFU of MVE was 100% in the

gld, *lpr* and wt mice. After inoculation of this high dose, clinical signs started to appear at day 4 in the wt and *lpr* mice, and at day 6 in *gld* mice. The average time to death was similar in the wt and *lpr* mice (days 5.5 and 5.8 pi, respectively), but was significantly delayed to day 6.8 pi ($P = 0.014$) in the *gld* mice. The similar pattern in the mortality rate of the *lpr* and wt mice may be due to the *lpr* mutation being 'leaky', whereby small amounts of functional Fas are expressed in those mutant mice (Booker et al., 1998).

To conclude, this part of the study indicates that the Fas pathway of cytotoxicity has no protective role in MVE infection.

Role of IFN-γ in recovery from MVE infection

NK/Tc cells also exert an antiviral function via a nonlytic pathway that involves cytokines (Ruby and Ramshaw, 1991; Biron, 1994). IFN-γ is secreted by T and NK cells and plays an important role in many antiviral immune responses (Ruby and Ramshaw, 1991; Young and Hardy, 1995). When I gave 6-week-old IFN-γR-/- mice a peripheral infection with a small dose of MVE iv (10^2 PFU), they showed a similar time to death of 11.6 days as the wt mice (comparative data for wt mice taken from Table 4.1), but displayed an increase in mortality (73%; $P = 0.44$) from encephalitis compared with the 46% of wt mice. This result suggests that the lack of the cytokine receptor enhances the susceptibility of mice to MVE.

6.3.2 Virus growth in tissues of wt mice and mice with a deficiency in either the granule exocytosis or the Fas pathway of cytotoxicity

I infected groups of perf-/-, gzmAxB-/-, perfxgzmAxB-/-, *gld*, *lpr* and wt mice with 10^2 PFU of MVE and groups of perf-/-, *gld*, *lpr* and wt mice with 10^5 or 10^8 PFU of MVE. The data for virus titers in organs of wt mice infected with 10^8 PFU were taken from chapter 4. I collected spleen, lymph nodes, muscle and brain on days 4, 6 and 8 after infection with 10^2 PFU, on days 2, 4, 6 and 8 after infection with 10^5 PFU, and on days 2, 3 and 4 pi after infection with 10^8 PFU of MVE.

Table 6.1 **Mortality of 6-week-old C57Bl/6 mutant and wt mice that are defective in the NK/Tc-cell granule exocytosis-mediated cytotoxicity, in the Fas-mediated cytotoxicity, or in IFN-γ receptor, following iv infection of MVE**

Dose (PFU)	Mouse strain	N[a]	% mortality[b]	ATD ± SEM[c]
0.1	wt	27	59	10.3 ± 0.6
	perf-/-	12	33	11.0 ± 0.7
	gzmAxB-/-	18	28	11.6 ± 0.6
	perfxgzmAxB-/-	6	33	11.0 ± 1.0
	gld	10	40	12.3 ± 1.5
	lpr	6	33	14.0 ± 0.0
10^2	wt	70	46	11.6 ± 0.4
	perf-/-	15	20	12.3 ± 2.2
	gzmAxB-/-	20	25	10.8 ± 1.5
	perfxgzmAxB-/-	19	32	12.5 ± 0.3
	gld	11	27	11.7 ± 0.7
	lpr	11	55	11.3 ± 1.1
	IFN-γR-/-[d]	22	73	11.6 ± 0.4
10^3	wt	9	56	12.0 ± 0.8
	perf-/-	16	38	12.0 ± 0.7
10^5	wt	20	40	10.9 ± 0.8
	perf-/-	12	33	13.3 ± 1.0
	gld	14	36	12.8 ± 1.0
	lpr	10	80	10.8 ± 0.6
10^8	wt	19	100	5.5 ± 0.2
	perf-/-	10	100	6.6 ± 0.2
	gzmAxB-/-	6	100	6.2 ± 0.4
	perfxgzmAxB-/-	5	100	9.4 ± 1.4
	gld	10	100	6.6 ± 0.3
	lpr	5	100	5.8 ± 0.2

[a] Number of mice infected.

[b] Mortality percentages are given for wt and mutant mice.

[c] The average time to death (ATD) was calculated in days ± standard error of the mean (±SEM).

[d] Mouse strain representing the cytokine effector mechanism of NK/Tc cells.

Kinetics of MVE replication in extraneural tissues of mice with a deficiency in the cytotoxicity pathways

Virus was not detectable (<10^3 PFU/g of wet tissue) in kidney or liver samples, or in blood (<10^2 PFU/ml) at any time, after infection with the low or medium doses in any of the mouse strains (data not shown), even though all the mice had seroconverted (Table 6.2). I only observed low levels of viremia at day 1 pi, after infection with 10^8 PFU of virus in all the mice tested (wt, *gld* and *lpr*); titers ranged from 10^2 to 10^3 PFU/ml. Virus detected in blood at this time might have been the remainder of high dose inocula, and disappeared at day 2 pi (data not shown).

When I tested spleen (Fig. 6.1), lymph nodes (Fig. 6.2), muscle (Fig. 6.3), serum, liver and kidneys (data not shown) by plaque assay, the results showed no significant differences between the different mouse strains. I detected spleen virus titers just above the limit of detection (10^3 PFU/g) when ko, mutant and wt mice were inoculated with the low (Fig. 6.1 A) or medium (Fig. 6.1 B) doses of MVE, but only in a few samples. After infection with 10^8 PFU of MVE, the virus titers in the spleen declined with time in all the mouse strains: virus load in all the mouse strains increased to 10^4 PFU/g at day 2 pi, and only half of the mouse groups tested positive (with 10^3 PFU/g) at day 4 pi (Fig. 6.1 C). MVE was detected in the lymph nodes in only two of 61 samples taken from the different strains (one in a *gld* and the other in an *lpr* mouse) after infection with 10^2 and 10^5 PFU of MVE (Fig. 6.2 A and B). After infection with 10^8 PFU of virus, titers gradually declined with time in lymph node samples of wt mice, but remained positive in most of the immunodeficient mice (Fig. 6.2 C). Virus titers in muscle were hardly detectable after infection with low to medium doses: only one perfxgzmAxB-/- and one gzmAxB-/- mouse had detectable virus titers at day 8 pi (after infection with 10^2 PFU), but none of the other 82 mutant or wt mice had

detectable virus titers. I detected virus in muscle mainly after infection with 10^8 PFU. Titers increased from 10^3 to 10^5 PFU/g from day 2 to 4 (Fig. 6.3 C). I did not observe a consistent difference in the growth kinetics, or in the values of the virus titers, in extraneural tissues, between the mice that are defective in NK/Tc-cell cytotoxic effector mechanisms and the wt mice. Given the apparent inability of extraneural tissues to support efficient growth of MVE, it is not possible to say whether: 1) NK/Tc-cell-mediated cytotoxicity contributes to the control of virus infection in these tissues, or 2) the level of virus titers in extraneural tissues is a factor that determines the incidence of neuroinvasion, and can thus account for the variability between individuals in the susceptibility to infection with MVE.

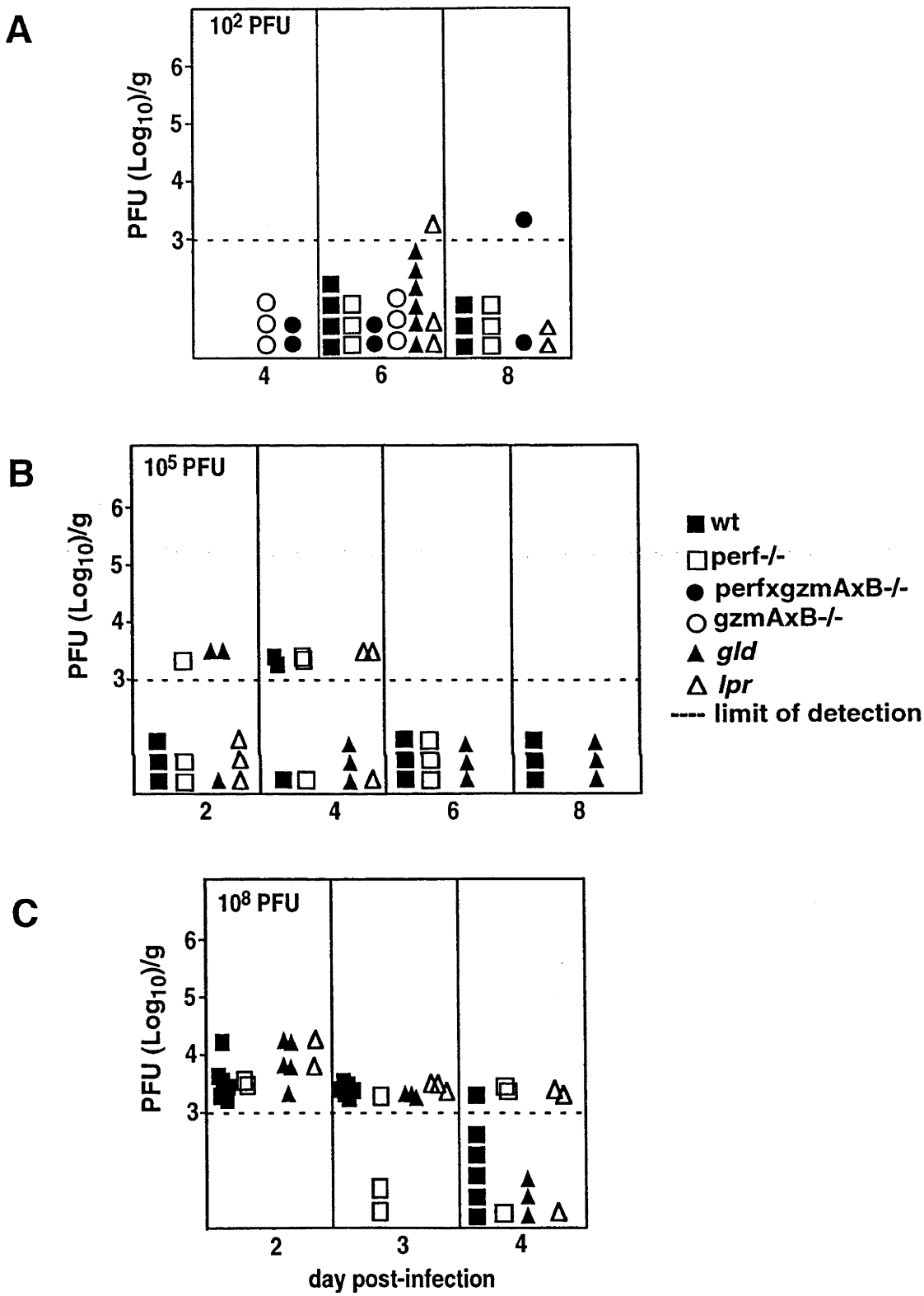

Fig. 6.1 Growth of MVE in spleen of mice with a deficiency in either the degranulation or Fas pathway of cytotoxicity. Mice were infected with 10^2 (**A**), 10^5 (**B**) and 10^8 (**C**) PFU of MVE iv. At the indicated times, animals were sacrificed and virus titers were determined by plaque assay. The lower limit of detection of virus titers was 1 x 10^3 PFU/g of wet tissue and is indicated by the interrupted line.

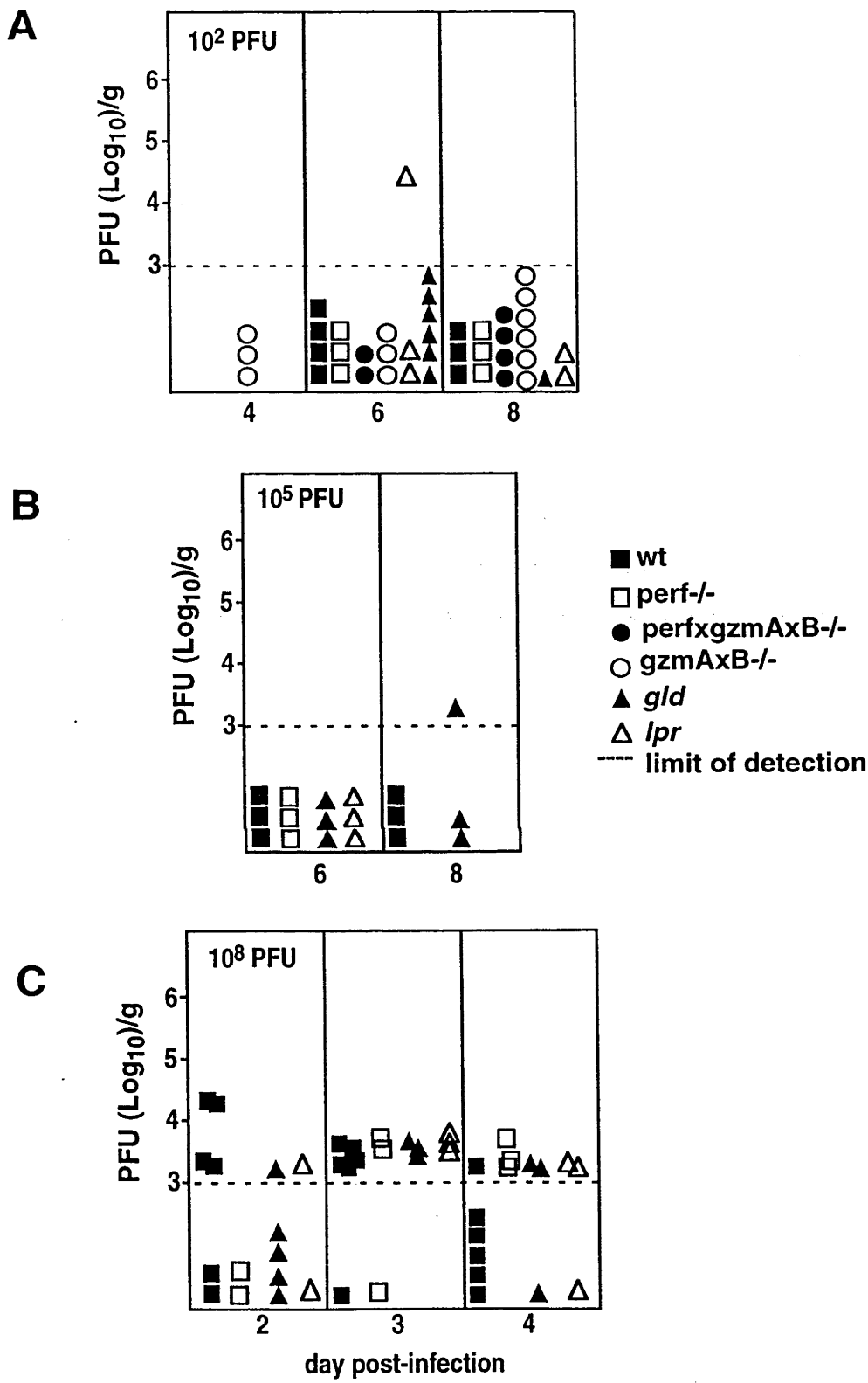

Fig. 6.2 Growth of MVE in lymph nodes of mice with a deficiency in either the degranulation or Fas pathway of cytotoxicity. Mice were infected with 10^2 (**A**), 10^5 (**B**) and 10^8 (**C**) PFU of MVE iv. At the indicated times, animals were sacrificed and virus titers were determined by plaque assay. The lower limit of detection of virus titers was 1 x 10^3 PFU/g of wet tissue and is indicated by the interrupted line.

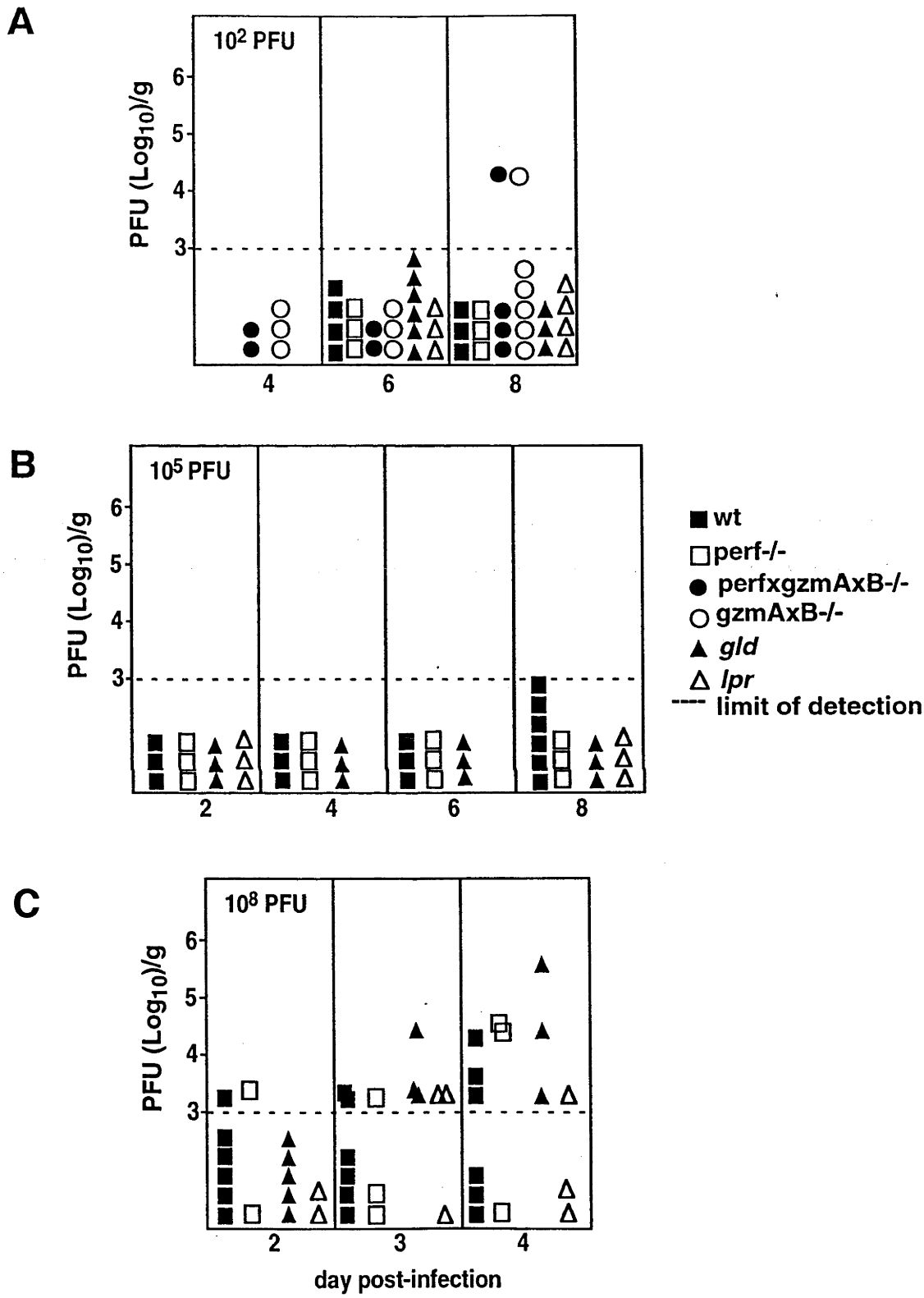

Fig. 6.3 Growth of MVE in muscle of mice with a deficiency in either the degranulation or Fas pathway of cytotoxicity. Mice were infected with 10^2 (**A**), 10^5 (**B**) and 10^8 (**C**) PFU of MVE iv. At the indicated times, animals were sacrificed and virus titers were determined by plaque assay. The lower limit of detection of virus titers was 1 x 10^3 PFU/g of wet tissue and is indicated by the interrupted line.

Kinetics of MVE replication in brains of mice with a deficiency in the cytotoxic pathways

To determine whether the reduced severity of the disease in the deficient mice was due to a reduced viral load in the brain, groups of 6-week-old perf-/-, gzmAxB-/-, perfxgzmAxB-/-, *gld*, *lpr* and wt mice were infected iv with 10^2 PFU of MVE, and a second group of perf-/-, *gld*, *lpr* and wt mice were infected with 10^5 or 10^8 PFU of MVE. I collected mouse brains at the same pi times as the extraneural tissues described above.

After inoculation of 10^2 PFU of MVE, I found low titers of virus from day 6 pi in brains of a few deficient mice of the *gld*, *lpr* and perfxgzmAxB-/- groups, but I did not detect virus in the brains of wt, perf-/- or gzmAxB-/- mice at this stage. By day 8 pi, I found virus in six out of eight wt mice, with titers that ranged from 10^4 to 10^8 PFU/g; in two out of six perf-/- mice, with titers of 10^7 PFU/g; in two out of four perfxgzmAxB-/- mice, with titers of close to 10^9 PFU/g; in four out of six gzmAxB-/- mice, with variable virus titers that ranged from 10^4 to 10^9 PFU/g; and in two out of six *gld* mice, with 10^7 to 10^8 PFU/g MVE titers (Fig. 6.4 A).

After inoculation of 10^5 PFU of MVE, none of the wt mice and only a few deficient mice showed detectable virus titers in the brain: on day 6 pi, one *gld* mouse showed a virus titer of 10^4 PFU/g; and on day 8, two perf-/- mice, all three *gld* mice and two out of three *lpr* mice showed virus titers that ranged from 10^3 to 10^9 PFU/g (Fig. 6.4 B). These results indicate that, after infection with low or medium doses of virus, the wt mouse brains show a delayed detection of virus and/or a reduction in virus titers compared with mutant mice. This happens despite the higher mortality of groups of wt mice, relative to mutant mice. This observation is valid until the last day of testing (day 8 pi).

Infection with 10^8 PFU of virus iv gave detectable virus titers at day 2 pi in brains of most animals: ranging from 10^3 to 10^6 PFU/g, and increasing to a value of 10^8 PFU/g on day 3 pi, and 10^9 PFU/g on day 4 pi in all the deficient mice, and in some of the wt mice. The mean titers of MVE in the brains of the four strains of mice were similar, although titers of wt mice showed more variation on days 3 and 4 pi, compared with the other groups (Fig. 6.4 C).

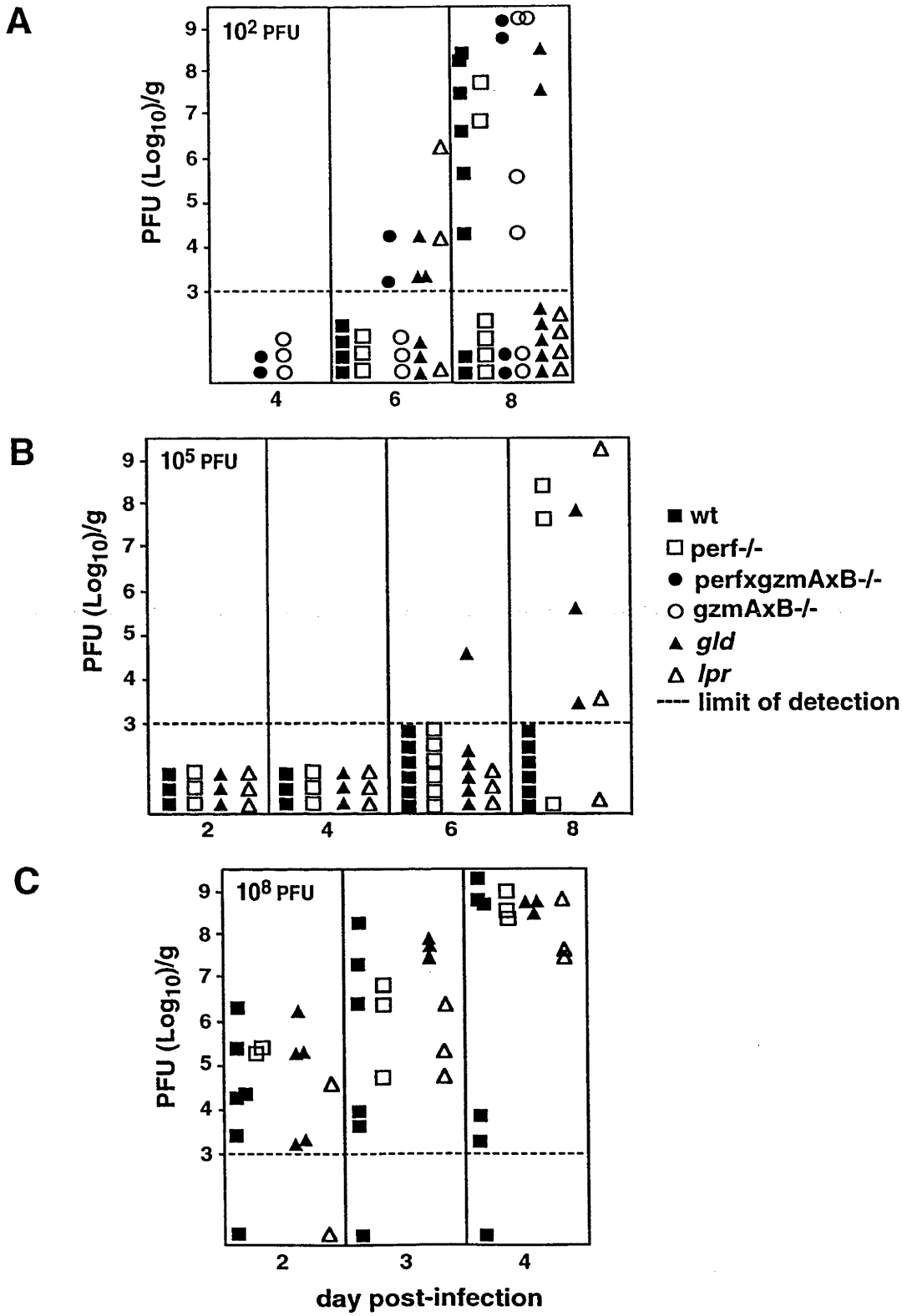

Fig. 6.4 Growth of MVE in brains of mice with a deficiency in either the degranulation or Fas pathway of cytotoxicity. Mice were infected with 10^2 (**A**), 10^5 (**B**) and 10^8 (**C**) PFU of MVE iv. At the indicated times, animals were sacrificed and virus titers were determined by plaque assay. The lower limit of detection of virus titers was 1 x 10^3 PFU/g of wet tissue and is indicated by the interrupted line.

6.3.3 MVE-specific antibody responses in wt mice and mutants with a deficiency in either the granule exocytosis or the Fas pathway of cytotoxicity

The humoral immune response against MVE in mice that are defective in either the granule exocytosis- or the Fas-mediated pathway of cytotoxicity was investigated to determine if the responses in these mice would differ (as had been reported for perf-/- mice by Sambhara et al. (1998)), and to verify if the mice had been productively infected. Given that vaccination of wt mice with 10^2 PFU (my results) or 10^6 PFU (Dr M. Lobigs) of UV-inactivated MVE does not induce MVE-specific Abs that are detectable by ELISA (data not shown), seroconversion of mice inoculated with live virus (0.1 to 10^5 PFU) was consistent with a productive infection (Table 6.2). Compared with the wt mice, there was a significant increase in the magnitude of the humoral immune response against MVE in some groups of *gld* mice, but not in the perf-/-, gzmAxB-/-, and *lpr* mice (Table 6.2).

The Ab levels in 9-week-old perf-/- and wt mice, after infection with 10^2 PFU of MVE iv, were also investigated. Sera collected at day 25 pi did not show a significant difference between the two mouse strains. The 9-week-old mice did not show an increase in anti-MVE Ab levels compared with the 6-week-old mice (data not shown); thus, the increase of resistance of the older mice may not be due to an enhanced Ab response.

Table 6.2 **MVE-specific Ab production in 6-week-old C57Bl/6 wt mice and mutant mice with a deficiency in either the granular exocytosis or the Fas pathway of cytotoxicity**

Mouse strain	Dose (PFU)	No./ group	Day pi	% Seroconversion	Mean Ab titer Log_{10} (range)[a]
wt	0.1	6	10	100	3.7 (2.6-4.1)
perf-/-	0.1	3	10	100	4.0 (3.8-4.1)
gzmAxB-/-	0.1	4	10	100	4.2 (4.1-4.4)
gld	0.1	3	10	100	3.7 (2.6-4.4)
wt	0.1	4	21-25	100	4.3 (3.8-4.7)
perf-/-	0.1	3	21-25	100	4.6 (4.1-5.0)
gld	0.1	1	21-25	100	5.0 (5.0)
wt	10^2	11	21-32	100	4.0 (2.9-4.7)
perf-/-	10^2	4	21-32	100	4.3 (4.1-4.4)
gzmAxB-/-	10^2	5	21-32	100	3.8 (3.5-4.1)
perfxgzmAxB-/-	10^2	8	21-32	100	4.0 (3.8-4.1)
gld	10^2	8	21-32	100	4.5 (3.5-5.0)[b]
lpr	10^2	4	21-32	100	4.3 (3.8-4.7)
wt	10^5	3	21	100	3.8 (3.5-4.1)
perf-/-	10^5	3	21	100	4.0 (3.8-4.1)
gld	10^5	3	21	100	4.5 (4.4-4.7)[b]

[a] MVE-specific antibody titers were determined by ELISA, and end points were calculated as described in Materials and Methods, Chapter 2.

[b] Statistically significant difference ($P \leq 0.05$; Mann-Whitney test) relative to values found in the groups of wild-type mice.

6.3.4 Histopathology

The results of histopathology and virus load determinations for brain and spleen samples of wt, perf-/-, *gld* and *lpr* mice, after infection with 10^5 PFU iv, are shown in Table 6.3.

Although histopathological changes and/or virus load in extraneural organs were rarely detected in the deficient mice after infection with 10^5 PFU of MVE, these were found more often in the mutant mice than in the organs of the wt mice. This corresponds with the observation that virus is more often present in tissues of the deficient strains (Table 6.3). Brains of the wt mice that were infected with 10^5 PFU of MVE yielded no virus when collected from day 2 to day 8 pi, which corresponds with the absence of histopathology. In contrast, the brains of perf-/-, *gld* and *lpr* mice yielded virus at day 6 and 8 pi, and showed histopathological lesions such as perivascular infiltration (pvi), meningitis and necrosis. Two of three brains from *lpr* mice showed meningitis on day 2 pi, and samples that gave rise to detectable virus at day 8 pi showed pvi in addition to meningitis (Table 6.3).

Table 6.3 Histopathology results of perf-/-, *gld*, *lpr*, and control wt mice infected with 10^5 PFU of MVE iv

		Brain		Spleen	
Mouse strain	**Days pi**	**Histology**	**Plaque assay**	**Histology**	**Plaque assay**
wt	2	normal	$<10^3$	reactive	$<10^3$
	2	normal	$<10^3$	normal	$<10^3$
	2	normal	$<10^3$	normal	$<10^3$
	4	normal	$<10^3$	normal	$1x10^3$
	4	normal	$<10^3$	normal	$<10^3$
	4	normal	$<10^3$	normal	$2x10^3$
	6	normal	$<10^3$	normal	nt
	6	normal	$<10^3$	normal	nt
	6	normal	$<10^3$	normal	$<10^3$
	8	normal	$<10^3$	normal	nt
	8	normal	$<10^3$	normal	nt
	8	normal	$<10^3$	normal	nt
Perf-/-	2	normal	$<10^3$	normal	$<10^3$
	2	normal	$<10^3$	normal	$<10^3$
	2	normal	$<10^3$	reactive	$<10^3$
	4	normal	$<10^3$	reactive	$<10^3$
	4	normal	$<10^3$	normal	$<10^3$
	4	normal	$<10^3$	normal	$<10^3$
	6	normal	$<10^3$	normal	nt
	6	normal	$<10^3$	normal	nt
	6	normal	$<10^3$	normal	nt
	8	extensive pvi, meningitis	$2.1x10^8$	reactive	nt
	8	extensive pvi, meningitis, necrosis	$3.4x10^7$	very reactive	nt
	8	pvi, meningitis	$<10^3$	reactive	nt
gld	2	normal	$<10^3$	reactive	$<10^3$
	2	normal	$<10^3$	normal	$<10^3$
	2	normal	$<10^3$	reactive	$<10^3$
	4	normal	$<10^3$	reactive	$<10^3$
	4	normal	$<10^3$	reactive	$<10^3$
	4	normal	$<10^3$	very reactive	$<10^3$
	6	normal	$<10^3$	normal	nt
	6	normal	$<10^3$	normal	nt
	6	normal	$<10^3$	normal	nt
	8	extensive pvi, meningitis	$7x10^7$	reactive	$<10^3$
	8	extensive pvi, meningitis	$2.5x10^3$	reactive	$<10^3$
	8	extensive pvi, meningitis	$1.8x10^5$	very reactive	$<10^3$
lpr	2	meningitis	$<10^3$	very reactive	$<10^3$
	2	meningitis	$<10^3$	reactive	$<10^3$
	2	normal	$<10^3$	normal	$<10^3$
	4	normal	$<10^3$	normal	$5x10^3$
	4	normal	$<10^3$	reactive	$5x10^3$
	4	normal	$<10^3$	normal	$<10^3$
	8	pvi, meningitis	$1.6x10^9$	reactive	nt
	8	normal	$<10^3$	reactive	nt
	8	extensive pvi, meningitis	$5x10^3$	slightly reactive	nt

normal: no histological lesions, similar to mock-infected mice (data not shown).
pvi: perivascular infiltration.
nt: not tested.
reactive: spleen reaction to antigens with the development of secondary follicles.

6.4 Discussion

In general, the mortality patterns in the mutant mice showed a similar trend as in the wt mice. There were two kinetically distinct processes that lead to fatal encephalitis depending on: 1) a high virus dose with 100% mortality and, 2) a low to medium dose, with close to 50% mortality (as discussed in Chapter 4). Although there was no statistically significant difference in the mortality rate from different doses between the different mouse strains, a general and surprising trend of reduced and delayed mortality was observed in the immunodeficient mice. Consequently, either the lack of expression of the main constituents of the cytolytic vesicles (perforin, granzymes A and B), or the deficiency in Fas ligand (*gld*) causes the enhancment of survival rates in mice that are infected with MVE.

Contrary to the above mutant mice, the FasR-deficient (*lpr*) mice and gamma interferon receptor knockout (IFN-γR-/-) mice mostly showed a higher mortality than the wt mice. For the *lpr* mice this may have two explanations. The first explanation is that *lpr* mice are 'leaky', which means that some functional Fas is still expressed (Booker et al., 1998). The second explanation is that *lpr* mice show infiltration of lymphoid cells, not only in lymphoid organs (which is more often evident in mice that are older than 6 weeks), but also into the choroids plexus. The latter may be a consequence of increased BBB permeability in this site (Vogelweid et al., 1991). The presence of a functional Fas receptor is also required for Fas-FasL-dependent suppression of inflammation (Griffith et al., 1995; Okuda et al., 1998; Park et al., 1998). Thus, in the *lpr* mice there may be an additional increase in the invasion of inflammatory elements, together with the virus, into the CNS. IFN-γ and NO have been reported as being important in the protection against YF encephalitis (Liu and Chambers, 2001) and JEV infection (Liu and Chambers, 2001; Saxena et al., 2001) in

animal models. However, there are also reports that suggest that depletion of macrophages (major producer cells of NO) may exacerbate WNV infection (Ben-Nathan et al., 1996). Recent studies of the role of IFN-γ and NO during infection with MVE suggest that their potentially deleterious inflammatory response is more than compensated by their antiviral effect (Lobigs et al., 2003). In my study, IFN-γR-/- mice succumbed in higher numbers than wt mice, when inoculated with 10^2 PFU of MVE. This suggests that the IFN-γ-receptor is important for the antiviral effect of the cytokine. Such effect may involve NO production, class-switching of immunoglobulin isotypes, and/or direct antiviral function in the CNS (Kundig et al., 1993; Binder and Griffin, 2001).

To determine if the absence of cell-mediated cytotoxicity alters the kinetics and the load of virions in the tissues, I assayed extraneural organs and brain virus load in groups of mutant and wt mice that had been injected with low to high doses of MVE. I found that the infection of mice with low (10^2 PFU) to medium (10^5 PFU) doses of MVE rarely gave rise to detectable virus in the extraneural tissues, that it never produced a detectable secondary viremia, and that all infected animals seroconvered. This confirms that there is productive virus infection present. In general, I recovered infectious virus from the tissues (predominantly spleen and brain) of immunodeficient mice at earlier times, and more frequently, than from the tissues of wt mice. This indicates that when the cytotoxic mechanisms are absent, neuroinvasion happens earlier. However, in general, wt mice still die earlier of typical viral encephalitis, and have higher numbers of dead mice, than the deficient mice.

The *gld* and granule exocytosis knockout animals used in this study showed, despite having earlier neuroinvasion and more (albeit slight) virus-positive tissue samples, a reduced mortality and a prolonged survival compared with the control wt

mice; which suggests that the presence of virus in the brain is not the only factor that contributes to disease. This suggests not only that cell-mediated cytolysis is important for the clearance of MVE, but also that it can contribute to clinical neuronal disease. These observations suggest that $CD8^{+}T$, and possibly $CD4^{+}T$ and NK cells, contribute directly to the morbidity and mortality of mice that have been infected with MVE. The lower mortality of mice with a deficiency in one of the two cytolytic pathways suggests that there is immunopathology after infection with MVE. It could be that lethal pathology is, at least in part, due to cytotoxic T cells that may induce a breakdown of the BBB. This, possibly MVE-triggered immunopathology, is, of course, hard to observe in earlier studies that mostly use younger mice of 3 weeks of age, because the immune Tc-cell response at that age is not mature enough to protect the animals. Consequently, the likelihood that an exaggerated pathological reaction is induced at that age is much less.

Contrary to the expectation, based upon established research in other virus models (Mullbacher and Flynn, 1996; Topham et al., 1997; Moskophidis and Kioussis, 1998; Mullbacher et al., 1999; Topham et al., 2001), that NK cells, Tc cells, and their cytotoxic mechanism are important in the control and clearance of infection, it may be that both the granule exocytosis and the Fas pathway of cytotoxicity can contribute to disease progression in MVE infection. However, a potentially over-compensatory function of the present cytotoxic mechanism, for the defective one cannot be discarded. Thus, in the next chapter, I continue this investigation with mice that are deficient in cytotoxicity of NK cells (beige mutation), or in both NK- and Tc-cell cytotoxicity (perforin and FasL mutations).

Chapter 7

Mice that are simultaneously deficient in perforin and FasL show an increased resistance to MVE infection

7.1 Summary

This chapter deals with disease outcome in the absence of NK- and Tc-cell cytotoxicity during an encephalitic flavivirus infection. I infected mice with a deficiency in NK-cell cytotoxicity (beige mutation) and mice that are simultaneously deficient in NK- and Tc-cell cytotoxicity (perf-/-x*gld* mutations) with MVE. Beige mice had a similar susceptibility to MVE infection like wt mice. This result confirms that NK cells have a limited role in encephalitic flavivirus infections, an observation that has been made by others. Mice that were simultaneously deficient in both pathways of NK/Tc-cell cytotoxicity — the granule exocytosis and the FasL/Fas (perf-/-x*gld*; Chapter 6) pathway — showed a higher resistance to MVE than syngeneic wt mice and mice with a single mutation (perf-/- or *gld*). This increased resistance of the double-deficient mice suggests that the cytotoxic mechanisms play a deleterious role in MVE infection.

7.2 Introduction

The role of antiviral cellular cytotoxicity in conferring protective or deleterious immunity during encephalitic flavivirus infections remains elusive. Mice that are deficient in both the degranulation and the Fas (perf-/-x*gld* mice) pathway are a unique tool to investigate cytotoxicity during infection. A limitation of the perf-/-x*gld* mouse model is that these mice develop a severe autoimmune syndrome. The Fas pathway plays a critical role in lymphocyte homeostasis, which accounts for the autoimmune syndrome observed in Fas-receptor *(lpr)* and Fas-ligand *(gld)* deficient mice. This disease is exacerbated in the absence of functional perforin (Nagata, 1999). Doubly-deficient mice are generated from the offspring of heterozygous perf+/-x*gld* progenitors, because homozygous perf-/-x*gld* mice cannot be established as a strain due to

lymphoproliferation in the female reproductive organs, and consequent infertility (Spielman et al., 1998). Additional to female infertility, male and female perf-/-x*gld* mice die of lymphoproliferation between 4 and 16 weeks of age, due to excessive mononuclear infiltrates in the pancreas, liver and kidneys (Kagi et al., 1999).

Up to now, perf-/-x*gld* mice have been used for the study of graft-versus-host disease (Braun et al., 1996; Martin et al., 1998; Jiang et al., 2001; Taylor et al., 2002), contact hypersensitivity (Kehren et al., 1999), inflammatory bowel disease (Simpson et al., 1998), vascular leak syndrome (Rafi et al., 1998), diabetes mellitus (Kreuwel et al., 1999), and chlamydiasis (Perry et al., 1999), but not as a model for flaviviral diseases.

NK cells are part of the innate immune system and are important for the early protective response against numerous virus infections (Kiessling and Wigzell, 1979; Herberman and Ortaldo, 1981; Hercend and Schmidt, 1988; Scott and Trinchieri, 1995; Biron et al., 1999). Only a limited number of studies have addressed the role played by NK cells in flavivirus infections (Vargin and Semenov, 1986; Kopecky et al., 1991; Lobigs et al., 1996; Momburg, 2001). In order to discriminate between the roles of cytotoxic and cytokine secretory functions of NK cells, in the recovery from encephalitic flavivirus infection, beige mice — which lack only the cytotoxic function — were used. Thus, in both beige and perf-/-x*gld* mice, cytokine secretion by lymphocytes is not deficient.

7.3 Results

7.3.1 Pathogenesis of MVE in mice with a deficiency in NK-cell cytotoxicity (beige mutation)

To assess the role of NK-cell cytotoxicity in the *in vivo* control of MVE infection, I used beige and wt mice of 6 weeks of age. The mice received a dose of 10^2 PFU of MVE iv, primarily for mortality studies. Groups of beige mice did not show significant difference in percent mortality compared with wt mice (60% and 45.5% respectively, $P = 0.69$) and ATD (10.5 and 11 days pi respectively; Table 7.1). I used three moribund beige mice (out of the 10 used for mortality studies) to assay virus load in the brain. Brains of beige mice, collected at day 11 pi, had titers between 10^7 and 10^8 PFU/g (data not shown). These values were similar to those obtained from brains of moribund wt mice that were collected at a similar time pi after infection with 10^2 PFU of MVE iv.

7.3.2 Perf-/-x*gld* mice die early due to lymphoproliferative disease

I observed mortality in a group of 21 non-infected doubly-deficient mice over a period of four months. The moribund mice had acute lymphoproliferation, especially in spleen and lymph nodes, as shown in Fig. 7.1, and died from as early as 4 weeks of age. Between 6 and 9 weeks, about one third of the doubly-deficient mice had died and by 13 to 14 weeks most of them had died of the lymphoproliferative disease (Fig. 7.2). At the start of the experiment, the six-week-old doubly-deficient mice had an average weight of 16.4 g, compared to 18.6 g for the syngeneic wt mice. At an age of 10 weeks, doubly-deficient mice had an average weight of 15 g, while the wt mice had increased their weight to 24 g (data not shown). Thus, the 10-week-old doubly-deficient mice

weighed on average 9 g less than the wt mice. Such a weight difference could make the doubly-deficient mice more susceptible to the MVE infection, because they would have more virus per body weight after inoculation with a similar dose. During the course of this experiment some perf-/-x*gld* mice showed signs of illness, such as a hunched back, ruffled fur and gradually increased emaciation.

It was important to know if mortality due to lymphoproliferative disease could be distinguished from mortality due to MVE infection. Ruffled fur and a hunched back are signs that are also present during MVE infection of mice. However, the clinical signs of MVE infection can still be distinguished from those of lymphoproliferative disease. One of the signs that characterize MVE infection is hind-leg paralysis, which never appears in the mice affected by lymphoadenopathy. Additionally, moribund MVE-infected mice mostly die approximately 24 h after the onset of the clinical signs. This is different to mice that suffer from lymphoadenopathy, which can survive with signs of lethargy, reduction in feeding and consecutive emaciation for weeks. Another difference is that mice affected by the autoimmune syndrome have lymph nodes and spleen that are up to 10 times bigger than the respective organs in mice that show symptoms of MVE infection (Fig. 7.2). Enlargement of lymph nodes and spleens in the double-deficient mice that die, following infection with MVE, could also be observed, although to a lesser extent. Finally, testing for MVE in brains of doubly-deficient mice is a further criterion to define if mortality was due to lymphoproliferative disease or to viral encephalitis.

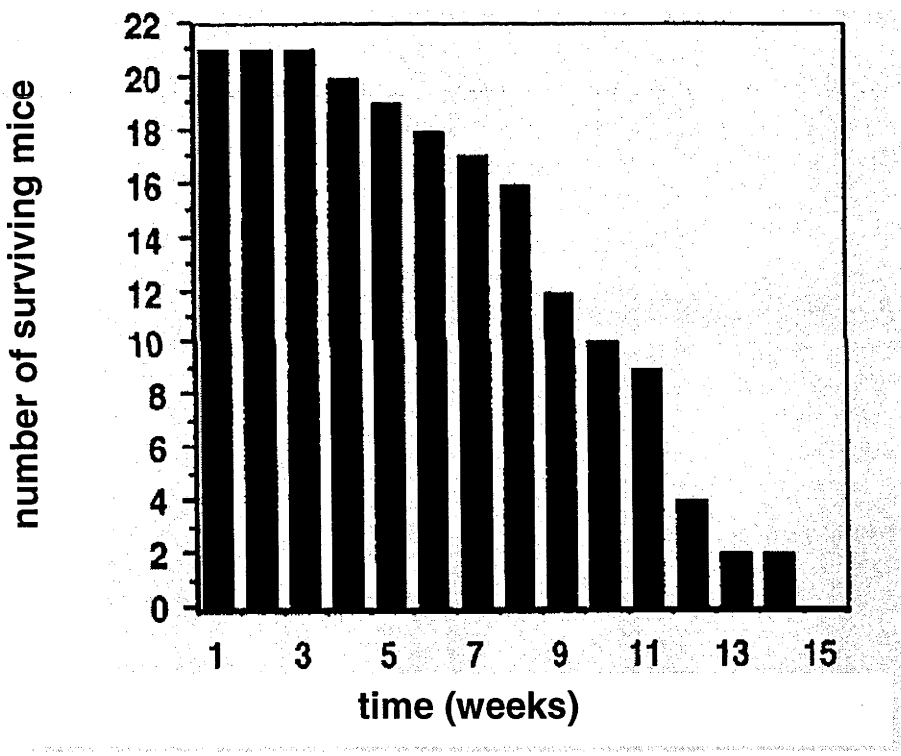

Fig. 7.1 Time to death of animals in a cohort of perf-/-x*gld* mice.

7.3.3 Resistance of mice that are doubly-deficient in granule exocytosis- and Fas-mediated cytotoxic pathways, to infection with MVE

A comparative study on the susceptibility of perf-/-x*gld* and wt mice to infection with MVE was of interest, to try to explain the apparent increase in resistance of mice that are deficient in the degranulation or Fas pathways of cytotoxicity to MVE (Chapter 6), as well as to investigate the possibility of redundancy between the two major pathways of cytotoxicity (Topham et al., 1997; Parra et al., 2000; Topham et al., 2001). The latter could have resulted in underestimation of the detrimental role of NK/Tc cells in encephalitic flaviviral disease.

Initially, I infected 9-week-old perf-*/-xgld* mice with 10^2 PFU of MVE iv and observed daily for 21 days. At 9 weeks, similar to wt mice, the double-deficient mice survived the MVE infection (Table 7.1).

Next, I infected the following mice with 10^2 PFU of MVE iv: 6-week-old mice that were homozygous for *gld* and heterozygous for perforin (perf+/-x*gld*), mice that were homozygous for perforin and Fas ligand deficiencies (perf-*/-xgld*), and wt mice. Surprisingly, the group of homozygous (perf-*/-xgld*) double-deficient mice did not show any cases of fatal viral encephalitis. The group of heterozygous mice (perf+/-x*gld*) had a marginally lower mortality than the wt mice: 33.3% and 45.5% respectively ($P = 0.42$) (Table 7.1). Four out of twelve doubly-deficient (perf-*/-xgld)* mice died without displaying the characteristic clinical signs of viral encephalitis. This mortality rate was consistent with that of an uninfected perf-/-x*gld* control group (Fig. 7.1).

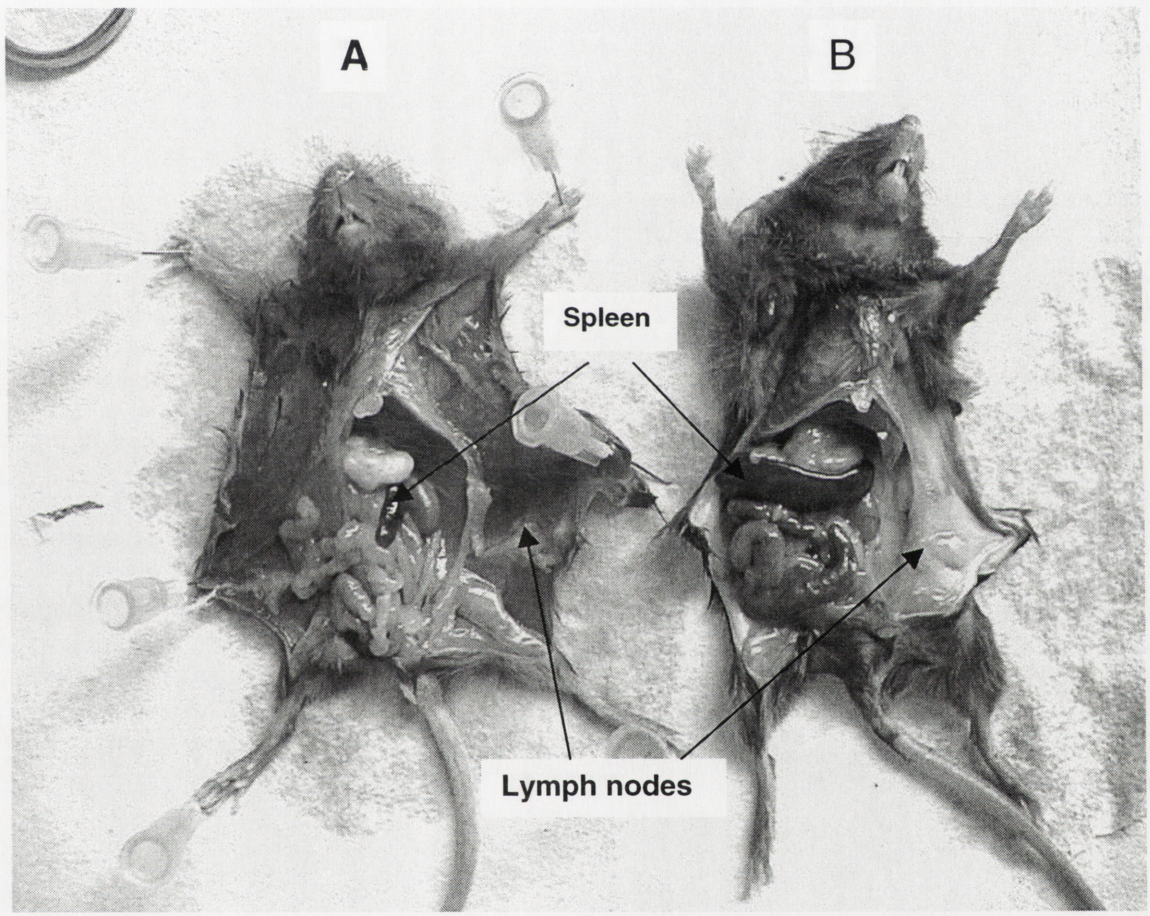

Fig. 7.2 Acute lymphoproliferative disease. Lymph nodes and spleen in a wt mouse (**A**), compared with a perf-/-x*gld* mouse (**B**); both mice are 10 weeks of age.

Because I could not recover MVE from the brains of the perf-/-*xgld* cadavers within a period of 16 h after death had occurred (data not shown), and because these mice showed greatly enlarged lymph nodes and spleens (Fig. 7.2), death was most likely due to lymphoproliferative disease.

After increasing the dose of MVE to 10^5 PFU, results confirmed the increased resistance in the perf-/-*xgld* mice. All eight perf-/-*xgld* mice survived without showing clinical signs of encephalitis; in contrast to 40% mortality in the wt group (Table 7.1).

After a high-dose infection with 10^8 PFU of MVE by the iv route, the 6-week-old perf-/-*xgld* and wt mice showed 100% mortality. The 10-week-old perf-/-*xgld* mice and wt mice had mortality values of 75% and 80%, respectively (Table 7.1). In these experiments, the mortality values for the doubly-deficient and wt mice were similar to each other, but the ATD was significantly longer in the doubly-deficient animals (7.3 compared with 5.6 days for 6-week-old mice [$P = 0.14$], and 9.7 compared with 7.0 days [$P = 0.14$] for the 10-week-old mice). After an intracranial injection of 10^2 PFU of MVE in 6-week-old perf-/-*xgld* and wt mice, I observed 100% mortality in both groups, a result similar to when a peripheral injection of a high dose of MVE was given. This result suggests that once the virus is in the brain of the doubly-deficient mice, similar percentages of mortality and ATD to those of wt mice occur (Table 7.1).

Results shown in Chapter 6 suggested that mice with functional defects in the granule exocytosis- or Fas-mediated pathway of cytotoxicity are less susceptible to a lethal infection with MVE than wt mice. Those results, together with the increased resistance, shown in this chapter, that occurs in mice that lack both pathways of

cytotoxicity (perf-/-*xgld*), give evidence that NK/Tc-cell mediated killing can contribute to the pathogenesis of encephalitic flaviviruses in their vertebrate hosts.

Table 7.1 Mortality as a consequence of iv infection with MVE in mice with a deficiency in NK cytotoxicity (beige mutation) and in mice with a deficiency in NK/Tc-cell cytotoxicity (perf+/-x*gld* and perf-/-x*gld*)

Dose (PFU)	Mouse strain [a]	Age (w)	Route	N[b]	% mortality	ATD ± SEM[d]
10^2	beige	6	iv	10	60.0	10.5 ± 0.3
	wt	6	iv	11	45.5	11.0 ± 1.1
	perf-/-x*gld*	6	iv	8	0.0[c]	-
	perf+/-x*gld*	6	iv	15	33.3	12.8 ± 1.8
10^2	wt	6	ic	3	100.0	6.0 ± 0.6
	perf-/-x*gld*	6	ic	3	100.0	6.3 ± 0.3
10^2	wt	9	iv	2	0.0	-
	perf-/-x*gld*	9	iv	5	0.0	-
10^5	wt	6	iv	20	40.0	10.9 ± 0.8
	perf-/-x*gld*	6	iv	8	0.0	-
10^8	wt	6	iv	5	100.0	5.6 ± 0.2
	perf-/-x*gld*	6	iv	6	100.0	7.3 ± 0.9
10^8	wt	11	iv	10	80.0	7.0 ± 0.5
	perf-/-x*gld*	10	iv	8	75.0	9.7 ± 1.5

[a] Morbidity and mortality of mutant and wt mice were recorded daily in all experiments and survivors were monitored for 21 days.

[b] Number of mice infected.

[c] Mortality due to lymphoproliferative disease occurred in the group of doubly-deficient mice on day 9 ($n = 1$) and 13 ($n = 3$), and was excluded from the total.

[d] The average time to death (ATD) was calculated in days ± standard error of the mean (±SEM).

7.3.4 Virus growth in tissues of perf-/-x*gld* and wt mice after iv MVE infection

Kinetics of virus growth in extraneural tissues

To assay for MVE in extraneural organs, I infected groups of double-deficient mice of 6 weeks of age with 10^2 or 10^8 PFU of MVE iv. Comparative data for the control wt mice were taken from Figure 4.3. When I tested spleen, lymph nodes and muscle of perf-/-x*gld* mice that were infected with a low dose of 10^2 PFU iv of virus at 4, 6 and 8 days pi, I did not detect virus in any of the tissues (data not shown). However, infection of the perf-/-x*gld* mice with a high dose of 10^8 PFU of MVE resulted in detectable virus titers in lymph nodes and muscle at 3 and 4 days pi. In contrast to wt, the perf-/-x*gld* mice did not give detectable virus titers in spleens (Fig. 7.3).

In general, virus was detected in less samples from extraneural organs of perf-/-x*gld* mice than wt mice. However, I noticed that lymph nodes and muscle titers of the doubly-deficient mice from day 4 pi were the highest among all the mutant and wt mouse strains tested, after infection with 10^8 PFU of virus.

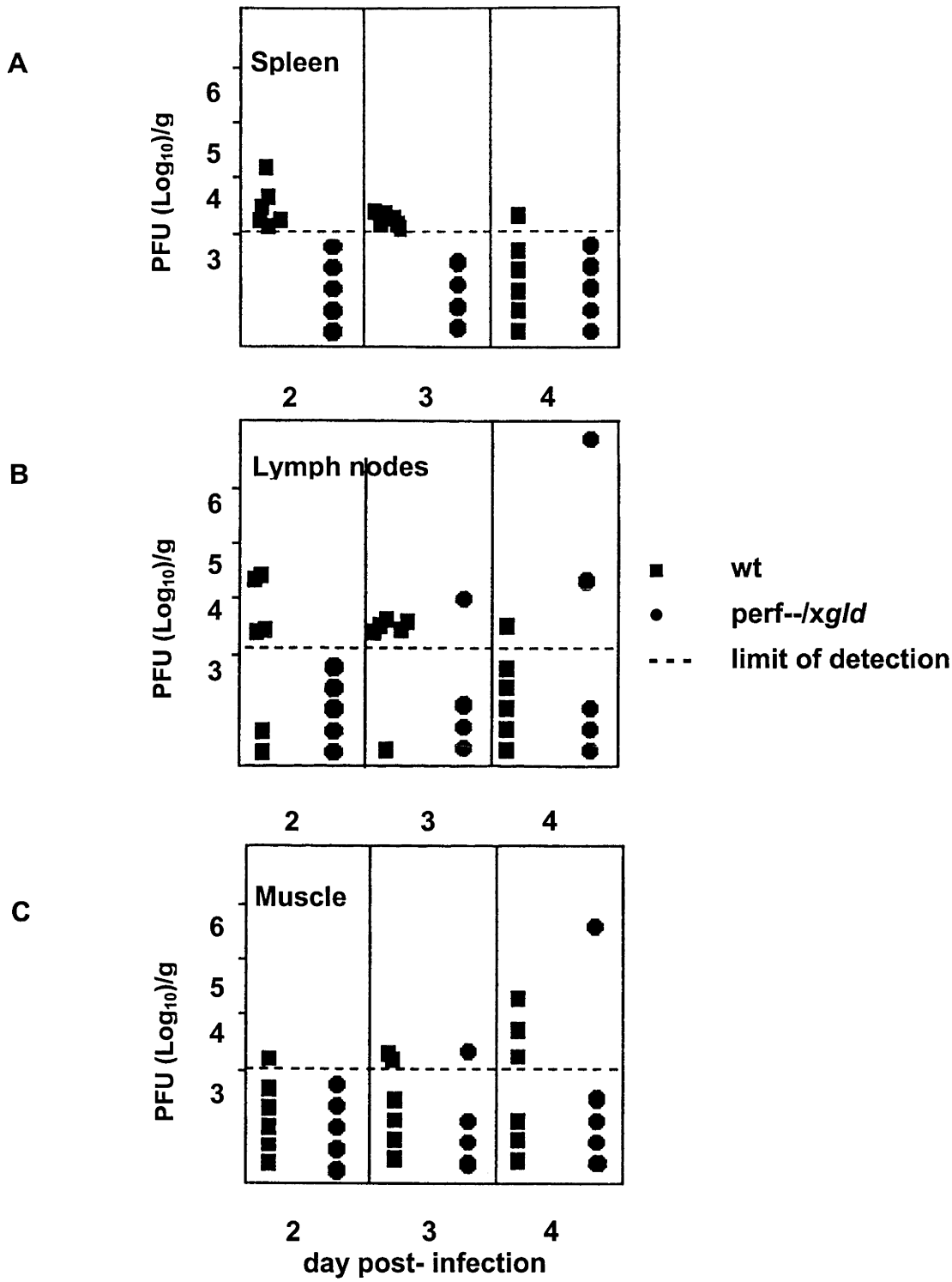

Fig. 7.3 Growth of MVE in extraneural tissues of mice with a deficiency in both subsets of the degranulation and the Fas pathway of cytotoxicity (perf-/-x*gld*) (●) compared with the wt mice (■). Six-week-old mice were infected with 10^8 PFU of MVE iv. At the indicated times, some animals were sacrificed, and virus titers in the spleen (**A**), lymph nodes (**B**) and muscle (**C**) were determined by plaque assay. The lower limit of detection of virus titers was 1 x 10^3 PFU/g of wet tissue, and is indicated by the interrupted line.

Kinetics of virus clearance from blood

The aim of this experiment was to test if the increased resistance to MVE infection in the perf-/-x*gld* mice was due to an improved capacity to clear virus from the blood. Spleen is considered to be the main organ that collects antigen from blood (Janeway, 1998). Spleen in perf-/-x*gld* mice is approximately 10 times enlarged, relative to spleen of wt mice, a condition which could facilitate the reduction of viremia in these mice relative to their wt counterparts. I infected six-week-old perf-/-x*gld* mice and wt controls with 10^8 PFU of MVE iv, and tested the level of viremia at 30 and 60 min pi. The kinetics of virus reduction from blood was similar in both strains of mice (Fig. 7.4 A). I also infected groups (n = 3) of 9-week-old doubly-deficient and wt mice with 10^8 PFU iv and collected serum at 2, 4, 6, and 9 hours pi. Virus titers in sera samples of perf-/-x*gld* mice were slightly higher, compared with those of the wt controls (Fig 7.4 B). These higher virus titers in the double-deficient mice may be due to their lower body weight. The similar levels of viremia found in the two mouse strains of 6 weeks of age suggest that there are factors, other than the increased capacity of virus clearance from the blood, present in the doubly-deficient mice which may account for the increased resistance against MVE infection. At day 2 pi, serum from three 9-week-old wt and perf-/-x*gld* mice showed no detectable MVE titers. The lack of detectable viremia at two days pi in the 9-week-old wt and doubly-deficient mice is consistent with the lack of detectable viremia in the younger 6-week-old mice, and with earlier findings of increased resistance to MVE infection in mice that are older than six weeks (as shown in Chapter 4).

Kinetics of neuroinvasion

To test if the reduced mortality in the doubly-deficient mice was due to a reduction in neuroinvasion, I injected six perf-/-x*gld* mice with 10^2 PFU of MVE iv and collected their brains at days 4, 6 and 8 pi. None of these brain samples showed detectable virus titers, which is consistent with a lack of encephalitic disease, and with death due to MVE infection (Fig. 7.5 A).

A higher dose of 10^8 PFU of MVE iv was also injected into doubly-deficient mice and their brains were tested for virus on days 2, 3 and 4 pi. Comparative data for the wt mice were taken from Chapter 4. At day 2 pi, brains of five out of six of the perf-/-x*gld* mice did not have detectable virus titers (the one positive sample had a titer of 1.4×10^3 PFU/g), but five out of six wt mice exhibited detectable virus titers of up to 2.4×10^6 PFU/g. At day 3 pi, all four brain samples of the perf-/-x*gld* mice showed titers that ranged from 3.5×10^6 to 2.5×10^8 PFU/g, while five out of six brains from wt mice had more variable titers, ranging from 6×10^3 to 1.3×10^8 PFU/g. At day 4 pi, all of five samples from the perf-/-x*gld* mice had titers that ranged from 7.3×10^7 to 5.6×10^9 PFU/g, and 5 out of 6 wt mice had variable titers that ranged from 1×10^3 to 2.7×10^9 PFU/g (Fig. 7.5 B). Thus, after iv inoculation with a high dose of MVE, the perf-/-x*gld* mice showed a delay in neuroinvasion of MVE, but at days 3 and 4 pi all mice showed virus growth in the brain and most of the virus titers were higher than those of the wt mice.

To further substantiate that virus growth in the CNS of the doubly-deficient mice was not inhibited by unknown factors, I also infected six-week-old perf-/-x*gld* and wt mice with 10^2 PFU of MVE ic. I collected brains at day 4 pi, and assayed these for virus load. The titers were similar in both mouse strains (Fig. 7.5 C), consistent with the result that the perf-/-x*gld* mice show a similar mortality than the wt mice when the virus

is inoculated directly into the brain, although the ATD was delayed by two days in the double-deficient mice.

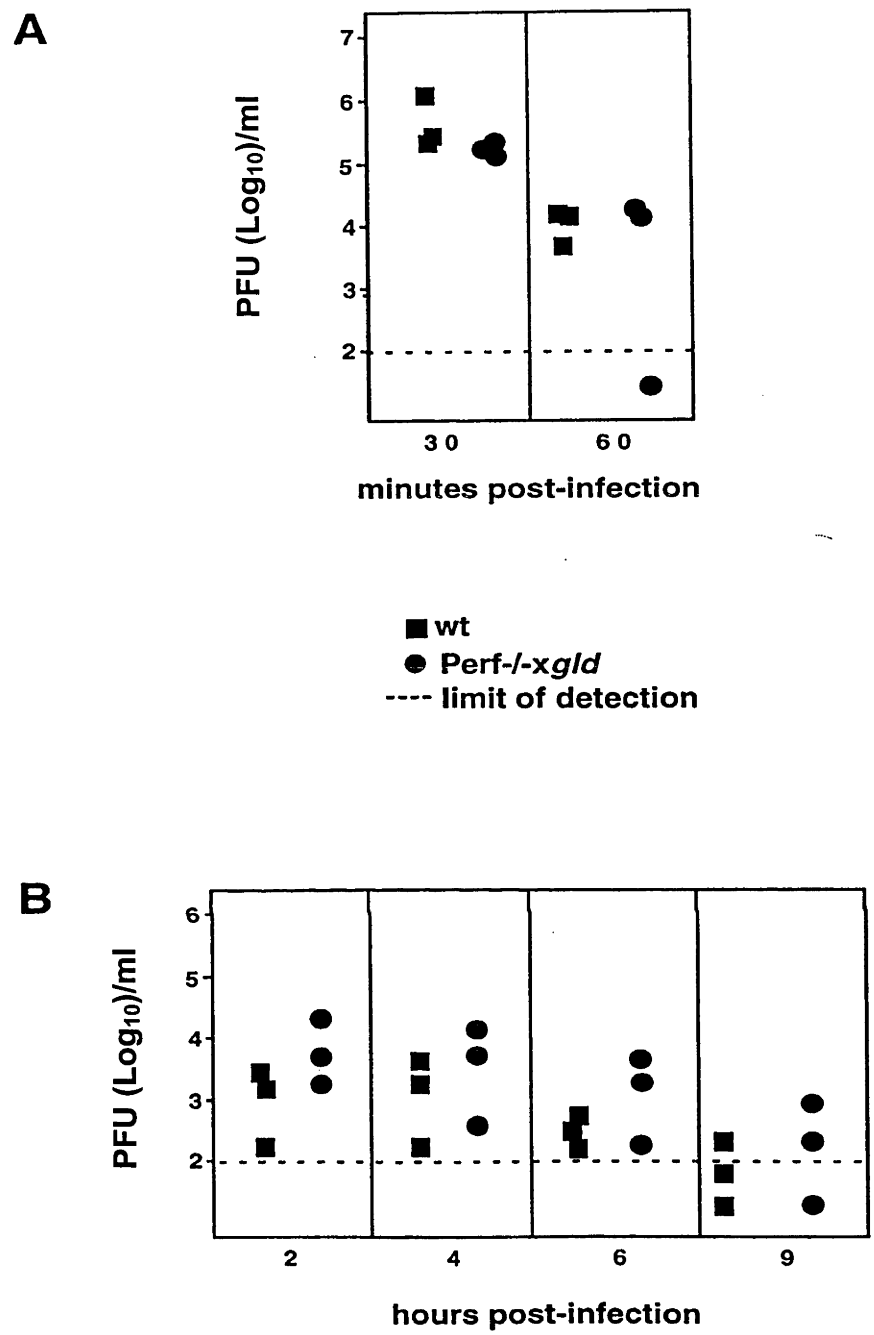

Fig. 7.4 MVE in serum of mice with a deficiency in both subsets of the degranulation and Fas pathway of cytotoxicity (perf-/-x*gld*) (●), compared to the wt mice (■). 6- (**A**) and 9-week-old (**B**) mice were infected with 10^8 PFU of MVE iv. At the indicated times, serum was collected and virus titers were determined by plaque assay. The lower limit of detection of virus titers was 1 x 10^2 PFU/ml, and is indicated by the interrupted line.

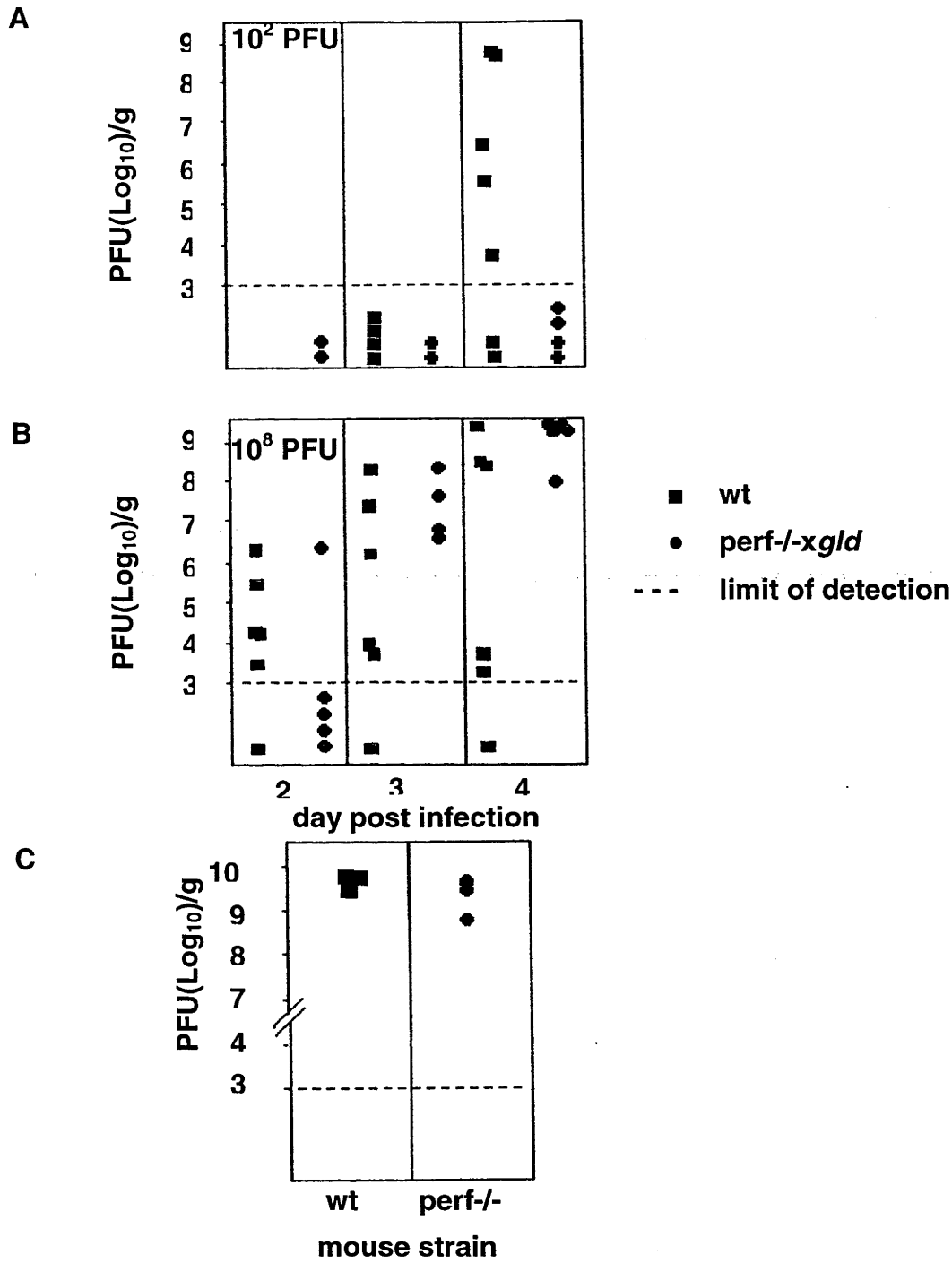

Fig. 7.5 Growth of MVE in brains of mice with a deficiency in both subsets of the degranulation and Fas pathway of cytotoxicity (perf-/-x*gld*) (•), compared to the wt mice (■). Six-week-old mice were infected with 10^2 (A), 10^8 (B) PFU of MVE iv and 10^2 PFU of MVE ic (C). At the indicated times, animals were sacrificed and virus titers were determined by plaque assay. The lower limit of detection of virus titers was 1 x 10^3 PFU/g of wet tissue and is indicated by the interrupted line.

7.3.5 The anti-MVE Ab response in perf-/-x*gld* mice

Another important factor that could contribute to the increased resistance of perf-/-x*gld* mice to MVE infection is the Ab response. I infected three or four perf-/-x*gld* mice, as well as wt control mice, with 10^5 PFU of MVE iv to test whether they show differences in the magnitude and kinetics of humoral immune response against MVE, and in incidence of seroconversion. At days 5, 10, 15 and 21 pi, I collected the sera and tested these for anti-MVE-Ab production by ELISA. The sera from all animals were reactive against MVE antigen from day 5 pi, and titers increased gradually until day 21. At day 5 pi titers were similar in both groups. A significant increase in the magnitude of the humoral immune response against MVE in perf-/-x*gld* mice, relative to wt mice, was seen at days 15 and 21 pi (Table 7.2).

Table 7.2 MVE-specific Ab production in 6-week-old C57Bl/6 perf-/-x*gld* and wt mice, after infection with 10^5 PFU iv

Mouse strain	No./ group	Day pi	% Seroconversion	Mean Ab titer,[a] log_{10} (range)
wt	3	5	100	2.7 (2.6-2.9)
perf-/-x*gld*	3	5	100	2.7 (2.6-2.9)
wt	4	10	100	3.3 (3.2-3.5)
perf-/-x*gld*	4	10	100	4.0 (3.8-4.4)[b]
wt	4	15	100	3.7 (3.5-3.8)
perf-/-x*gld*	4	15	100	4.1 (3.5-4.7)
wt	4	21	100	3.8 (3.8-4.1)
perf-/-x*gld*	4	21	100	4.5 (4.1-4.7)[b]

[a] MVE-specific antibody titers were determined by ELISA, and endpoints were calculated as described in Materials and Methods, Chapter 2.

[b] Statistically significant difference ($P \leq 0.05$; Mann-Whitney test) relative to groups of wt mice.

7.4 Discussion

The importance of Tc and NK cells in the control and clearance of infection in several virus models is well recognized (Blanden, 1971; Zinkernagel, 1993; Kagi et al., 1994; Mullbacher and Flynn, 1996; Mullbacher et al., 1999). Mice that lack NK cytotoxicity (beige mutation) and mice that lack both NK- and Tc-cell cytotoxicity (perf-/-x*gld*) provide an important *in vivo* model for the study of the role of cytotoxicity during viral diseases, which has not been thoroughly investigated in the case of encephalitic flaviviruses.

In this study, I found no significant difference in susceptibility to MVE between beige mice and wt mice. Previous studies suggested that NK cells play only a minor role in the control of flavivirus infection (Ilyinskikh et al., 1990; Hill, 1993; Lobigs et al., 1996; Momburg, 2001). Hill and Momburg (Hill, 1993; Momburg, 2001) hypothesized that the limited NK-cell activity during flavivirus infection is due to flavivirus-mediated MHC class I upregulation. In other words, the engagement of the NK-cell receptor with MHC class I results in a down-regulatory signal of NK-cell activity. Following this hypothesis, flavivirus-infected cells may employ this strategy to escape NK-cell lysis, and to produce sufficient viremia, that can be transmitted to an arthropod vector, in the natural host (mainly birds) during early infection (Momburg, 2001).

My work on mice that lack either the degranulation or the Fas pathway of cytotoxicity (Chapter 6) showed that those cytotoxicity mechanisms do not play an essential protective role during MVE infection. Because of the possibility of redundancy between the two major pathways of cytotoxicity (Topham et al., 1997; Parra et al., 2000; Topham et al., 2001), a potentially deleterious role of NK/Tc cells in MVE infections cannot be defined when using mice that are only defective in one of the two pathways. Thus, mice that lack both cytotoxic mechanisms were generated. Studies

described in this chapter show that perf-/-x*gld* mice are significantly less susceptible to MVE than both the wt mice, and the mice with a deficiency in only one pathway of cytotoxicity. The greatly increased resistance of perf-/-*xgld* mice to MVE infection suggests that immunopathology is the cause, since NK/Tc-cell-mediated killing seems to contribute to the pathogenesis of encephalitic flaviviruses in their vertebrate hosts.

The cytotoxic mechanisms do not account for the capacity of the mice to clear MVE infection from extraneural organs. Most of the extraneural organs from the doubly-deficient mice have similar, or even lower, virus titers than those of wt mice. However, the possibility that the brains of perf-/-*xgld* mice are resistant to replication of the virus can be excluded: when a low dose of MVE is injected directly into the brain, or when a high dose is injected iv, all doubly-deficient animals, like the wt controls, develop fatal encephalitis, with virus titers in the brains similar to those in wt mice.

The 100% seroconversion in all mutant and wt mice that are MVE-infected confirms that infection and extraneural growth of MVE occurs in these animals. The humoral immune response against MVE was elevated (relative to wt mice) in mice with the *gld* mutation only (Chapter 6), and also in the doubly-deficient perf-/-x*gld* mice (this chapter), but not in mice with a targeted disruption of the perforin gene (Chapter 6). The latter contradicts reports on other viral infections (Sambhara et al., 1998); however, given that the perf-/-*xgld* mice were significantly more resistant to MVE infection than the *gld* mice, it is unlikely that antibody-mediated virus clearance can account for the greatly reduced mortality in the former (Table 7.2).

What is the mechanism by which a deficiency in the two main cytotoxic effector functions prevents fatal flaviviral encephalitis? It is conceivable that cytotoxic cells disrupt the BBB by killing infected endothelial cells that line the brain capillaries, thus allowing virus access into the brain parenchyma. When the encephalitic flavivirus WN

infects human endothelial cells, the expression of leukocyte adhesion molecules is induced at the plasma membrane of the infected cells (Shen et al., 1997), making them susceptible to NK/Tc cell-mediated attack. The kinetics of appearance of MVE in the brain (at 6 to 8 days pi) following iv infection with a low virus dose, is consistent with that of the induction of the anti-viral Tc-cell response (Kesson et al., 1987) and, in turn, with the Tc-cell-induced damage of the cells that line the brain capillaries.

More work has to be done to produce direct experimental evidence to support the hypothesis that damage to the integrity of the BBB occurs because the cytotoxic effector pathways allow encephalitic flaviviruses to invade the brain. The possibility that a third NK/Tc cell antiviral effector pathway, namely cytokines, could account for the increased protection that is seen in perf-/-x*gld* mice also has to be studied in the doubly-deficient mice. However, work done by Perry et al. (1999) shows that perf-/-x*gld* mice, infected with *Chlamydia*, do not show statistically significant differences between the levels of IL-6, IL-10 and IFN-γ in wt, *lpr*, *gld*, perf-/- and perf-/-x*gld* mice.

Few studies have been done using the perf-/-x*gld* mice, possibly due to the great amount of work involved to obtain sufficient numbers (which involves backcrossing of heterozygous perf+/-x*gld* mice, defining the genotype, and high levels of mortality due to the lymphoadenopathy when perforin and FasL are missing). Cretney et al. (2002) performed studies that show that the inflammatory mediator TNF contributes to the immunopathology of perforin/Fas-ligand double deficiency. The Cretney group compared disease progression in perf-/-x*gld* mice that were also deficient in TNF (triply-deficient TNFxperf-/-x*gld* mice). Unlike the perf-/-x*gld* doubly-deficient mice, triply-deficient mice were comparatively fertile, with the majority of mice not suffering severe pancreatitis or hysterosalphingitis in the first five months of life. However, at 8 weeks of age, TNFxperf-/-x*gld* mice developed pancreatitis, which suggests that loss of

TNF was not completely protective. That study concludes that TNF is necessary for the full manifestation of immune deregulation caused by the perforin/FasL-deficiency, and, in particular, is responsible for the overwhelming early tissue infiltration and destruction caused by inflammatory cells.

Whenever doubly-deficient mice have been used in disease studies (see section 7.2) they have shown a reduced disease outcome; a result that is likely due to the reduction of vascular leak syndrome and inflammation. Inflammation and vascular leakage are some of the most widely noted phenomena in a variety of clinical diseases. Although the BBB has not been studied in the doubly-deficient mice in combination with viral agents, vascular leakage could (at least in the case of encephalitic flavivirus infections) be the cause of virus-activated NK/Tc cytotoxic damage to the BBB, and lead to encephalitis. Most of the clinical diseases caused by flavivirus (if not all) have an inflammatory and sometimes hemorrhagic (e.g. dengue hemorrhagic fever) presentation. Could the lack or reduction of perforin and FasL expression reduce the severity of other flaviviral diseases? Or could it reduce the severity of other inflammatory-hemorrhagic pathologies?

Chapter 8

Concluding remarks

The major aims of the studies presented in this thesis were two-fold: 1) to establish a mouse model for the study of MVE pathogenesis that parallels important aspects of the human disease, and 2) to investigate whether cytolytic lymphocytes give protection against, or exacerbate, the encephalitic disease.

Mice provide an excellent animal model for flaviviral encephalitis in humans, given that infection with members of the encephalitic flaviviruses mimics the low or absent viremia, the lack of detectable virus growth in extraneural tissues, and the low but mostly lethal incidence of CNS infection found in humans. However, although consistent with the human disease, the low incidence of CNS invasion in adult mice that are infected by an extraneural route demands the use of large numbers of animals in order to undertake studies into the pathogenesis of these viruses. To avoid this problem three- to four-week-old mice that are highly susceptible to extraneural flavivirus infection have been used in many virulence and pathogenesis studies to date, for instance: MacDonald, 1952; Huang, 1963; Cole and Wisseman, 1969; Monath et al., 1980; Hase et al., 1990; Lobigs et al., 1990; Cecilia and Gould, 1991; Mason et al., 1991; Hasegawa et al., 1992; McMinn et al., 1996; Guirakhoo et al., 1999; Broom et al., 2000; Gritsun et al., 2001; Chambers et al., 2003; and Gupta et al., 2003.

The high lethality of encephalitic flavivirus infection in young mice is thought to be the result of the immaturity of their immune system and a blood–brain barrier (BBB) that is not fully developed. Hence, it is clearly important to use animals with fully developed immune systems for the study of immune responses against flavivirus infection. To date, few pathogenesis studies of JEV and MVE have used

immunologically mature adult mice of at least six weeks of age. These include studies by MacDonald (1952), Huang (1963), Mathur et al. (1983), Lange and Sedmak (1991), Lad et al. (1993), my own work (Licon Luna, 2002), and subsequent work by members of our research group (Lee and Lobigs, 2002; Lobigs et al., 2003a; Wang et al., 2004).

In this thesis C57Bl/6 adult mice of at least six weeks of age were used to establish pathogenesis parameters for the prototype strain of MVE. Parameters included mortality, the virus growth in brain and extraneural tissues, the kinetics and magnitude of the virus-specific antibody response, the induction of a virus-specific Tc-cell immune response, and histopathological manifestations. The C57Bl/6 inbred mouse strain was chosen because it made a wide range of mice with gene knock-out and null mutations available and gave rise to mice with defined defects in humoral and cellular immunity.

The pathogenesis studies showed that the age of the mice and the dose of virus were the variables that most influenced susceptibility to MVE infection. Mice of 10 weeks of age were considerably more resistant than mice of six weeks of age. Thus, mice of the latter age-group were used for most of the studies, given that a subset of approximately 50% of these mice developed encephalitis and succumbed after infection with low to medium doses of MVE. Mice were mostly infected iv, to mimic the natural route of infection by hematophagous mosquitoes. An important finding of the studies (see Chapter 4) was the lack of a dose response over a wide range of MVE inocula ranging from 0.1 to 10^5 PFU, each dose giving a similar percentage of mortality at around day 11 pi. This disease pattern suggests that the virus first replicated in extraneural tissues prior to entering the CNS, and that invasion of the brain was a stochastic process unrelated to the dose of virus injected over a 10^6 range of iv virus inocula. A similar observation was made subsequently for the Sarafend (Wang et al., 2003b) and New York isolates of WNV (Diamond et al., 2003b). However, when a high

dose of 10^8 PFU of MVE was inoculated iv, 100% of mice died at ~day 6 pi. Given that the death rate and time to death of mice injected ic with a low dose (10 PFU) were virtually identical to those of mice that were injected iv with a high virus dose, it is most likely that the latter inoculation allowed virus in the inoculum to breach the BBB directly from blood. In contrast to iv infection with 10^8 PFU, infection with the lower doses delayed detectable neuroinvasion of MVE and ATD by approximately five days. This strongly supports the view that a period of extraneural virus replication preceded the spreading of the virus into the CNS.

Two fundamental questions on encephalitic flavivirus pathogenesis in humans and mice remain elusive, i.e.: 1) which extraneural tissue(s) supports virus growth prior to CNS invasion; and 2) by which mechanism(s) does virus spread into the CNS take place. My earlier findings (Licon Luna, 2002) show that there are two distinct disease progressions that depend on the virus dose. These are: an immediate presence of virus in the brain and an earlier occurrence of death (day 5–6 pi) in mice that are infected with a high ($\leq 10^8$ PFU) virus dose, and a later occurrence of virus in the brain combined with a variable mortality (after day 10 pi) in mice are infected with a low (10^5 PFU) virus dose. This difference in disease pattern strongly supports the view that in the latter scenario virus growth in extraneural tissues occurs prior to entry into the brain. It also raises the possibility that more than one mechanism may be involved in virus breach of the BBB. These candidate routes of neuroinvasion could be: 1) by the neuronal route after infection of the peripheral nerves; 2) by infection of highly susceptible olfactory neurons, which are unprotected by the BBB (Monath et al., 1983); 3) by virus entry into the vascular endothelial cells of capillaries in the brain, transcytosis, and release of virus into the brain parenchyma (Lad et al., 1993); and 4) by diffusion of virus between capillary endothelial cells in individuals that display a leakiness of the BBB due to

factors unrelated or secondary to the viral infection (Kobiler et al., 1989; Hase et al., 1990; Lustig, 1992).

Attempts to determine extraneural sites and kinetics of MVE growth in adult mice after iv infection with low to medium doses gave disappointing results. This may be due to technical problems that cause virus degradation during tissue preparation, and/or to the notion that flaviviruses only need to be present at concentrations under the limit of detection before they reach the brain. The use of two standard techniques (plaque assay and RT/PCR) on tissue samples to detect MVE addressed the possibility that different organs may contain different factors that affect the infectivity of the virus or the integrity of the viral RNA. One could speculate that the RT/PCR method is more sensitive because it can also detect the genomes of inactivated virus. However, both RT/PCR and plaque assay gave similar low efficiency of virus detection. Whilst conventional and even new technology of real-time RT/PCR (Mackay et al., 2002) can (in the latter case quantitatively) detect genetic material of the virus, these methods do not allow researchers to draw conclusions about the infectivity of the virus detected (Bae et al., 2003).

Muscle was the only extraneural tissue tested that harbored gradually increasing virus growth pi (Fig. 4.2), albeit at low levels. Since MVE seems to be predominantly neurotropic, its occurrence in muscle could have been the result of virus growth in nerve tissue (e.g. the large femoral nerve), which was not separated from the muscle tissue. Thus, the question whether virus growth occurred in muscle or in peripheral nerve tissue remains unresolved.

The 100% seroconversion of the mice infected with doses of MVE as low as 0.1 PFU is surprising in the light of poor virus growth in extraneural tissues. It suggests that either: 1) the two detection methods underestimated the virus content in tissues, or 2)

that a peripheral tissue that was not included in the screening allowed more efficient growth (albeit not reflected in detectable viremia) of MVE than the tested tissues, or 3) that a low, mostly undetectable, virus growth in extraneural tissues over a number of days is sufficient for CNS invasion to occur (either stochastically or with the additional requirement of insult to the integrity of the BBB). It is noteworthy that MVE and other encephalitic flaviviruses are rapidly removed from the bloodstream (MacDonald, 1952; Huang, 1963; Lee and Lobigs, 2002) and prevent accumulation of virus in the circulation (see Chapter 4).

Several researchers have used adult mice to study the pathogenesis of WNV, another member of the JEV serocomplex (Diamond et al., 2003b; Diamond et al., 2003a; Diamond et al., 2003c; Wang et al., 2003a; Wang et al., 2003b). One difference between WNV, MVE and JEV is that WNV can be isolated from the blood of human patients as well as from mice during the acute phase of infection, MVE and JEV usually cannot (MacDonald, 1952; Huang, 1963; Monath, 1996; Lee and Lobigs, 2002; Diamond et al., 2003b; Lobigs et al., 2003b). This suggests that there is more efficient growth of WNV in extraneural tissues. Furthermore, although WNV can also produce encephalitis, its most characteristic symptomatology resembles that of dengue-like fever (Hubalek and Halouzka, 1999; Sampson et al., 2000). Of relevance for the studies presented in this thesis is the finding that the murine $CD8^+$ Tc-cell response against WNV plays an important role in protection from disease (Shrestha et al., 2003; Wang et al., 2003b, 2004), whilst its role in MVE is deleterious. Thus, although there are many similarities in the pathogenesis of members of the JEV serocomplex flaviviruses, caution must be applied when extrapolating findings from one virus to another (Wang et al., 2004).

Published work on the role of Tc cell immune responses during encephalitic flavivirus infections (JEV, WNV, YFV, TBE) in the past resulted in conflicting findings: some researchers showed that the Tc-cell response was protective (Mathur et al., 1983; Murali-Krishna et al., 1996; Wang et al., 2003b) or had no apparent effect on the outcome of the disease (Pan, 2001), whilst others demonstrated that immunosuppression of cellular immunity enhanced the survival of mice from flavivirus infection (Hirsch and Murphy, 1968; Camenga and Nathanson, 1975; Semenov, 1975; Semenov et al., 1975). However, given the severity of some of those challenge models (ic inoculation of virus or disruption of the BBB), and the fact that the immunosuppressant drugs they used did not allow them to discriminate between the role of $CD4^+$ and $CD8^+$ T cell responses, the importance of the Tc-cell response in MVE infection of mice remained unknown.

This thesis demonstrates and partially characterizes the generation of an MVE-specific, H-2^b-restricted, $CD8^+$ Tc-cell response. This work will be fundamental for the subsequent work that investigates the role of cytolytic effector cells in the recovery from, or exacerbation of, MVE in C57Bl/6 wt mice. The H-$2D^b$-restricted response is mapped to the prM protein, which is somewhat unusual, although not unprecedented (Konishi et al., 1998; Co et al., 2002; van der Most et al., 2002), given that flavivirus-immune Tc-cell responses in mice and humans predominantly recognize determinants derived from the non-structural proteins, in particular NS3 (Hill et al., 1992; Rothman et al., 1993; Lobigs et al., 1994; Rothman et al., 1996; Lobigs, 1997; Spaulding et al., 1999).

A most surprising finding in my investigation on the role of cytotoxic effector function of NK/Tc cells in MVE infection is that mice which lack both the granule exocytosis- and the Fas-mediated cytolytic mechanism show a significantly increased

resistance relative to wt mice. This was most apparent when a low dose (10^2 PFU, iv) of MVE was used: 100% of perf-/-x*gld* mice survived, compared to 80% of perf-/- mice, 73% of *gld* mice, and 54% of wt mice. Two (not necessarily exclusive) interpretations can be forwarded to account for the difference: 1) that an anti-viral function is up-regulated in the double-deficient mice, preventing extraneural virus growth sufficient for neuroinvasion of MVE to occur, or 2) that the cytotoxic function of NK/Tc cells is detrimental for the host in MVE infection, for instance by causing leakage of the BBB. However, given the lymphoproliferative disease in perf-/-x*gld* mice, their levels of Ab, interferon, and cytokine could have been elevated relative to wt mice. In my experiments, the level of MVE-reactive antibodies was indeed higher in most of the perf-/-x*gld* mice than that in the wt and perf-/- mice, but similar to the level in *gld* mice. Given that the perf-/-x*gld* mice were significantly more resistant than the *gld* mice, it is unlikely that the higher level of antibodies accounted for the great increase in resistance of the double-deficient mice. In the absence of a systematic quantitation of interferon and cytokine production in perf-/-x*gld* mice, a contribution of these factors to the resistance of the mice to MVE infection cannot be excluded. One investigation (Perry et al., 1999) found that perf-/-x*gld* mice infected with Chlamydia do not have an increased level of IL-6, IL-10 and IFN-γ.

The reduced mortality I found in the double-deficient mice was not due to the inability of MVE to replicate in the brains of these mice because a low dose of virus (10^2 PFU) injected ic produced fatal encephalitis with virus titers in the brains of infected perf-/-x*gld* mice; a result that is comparable to that of wt mice. Could virus-immune Tc-cell-mediated lysis of endothelial cells that forms the BBB facilitate the entry of MVE into the brain? It is known that WNV can infect human endothelial cells (Shen et al., 1997) and that these cells express leukocyte adhesion molecules, making

them susceptible to NK/Tc-cell-mediated attack. Additionally, in my experiments, the time of the first appearance of MVE in the brain of wt mice, after they were inoculated with a low iv dose of the virus, was six to eight days pi. This time frame is consistent with the induction of an anti-flaviviral Tc-cell response (Kesson et al., 1987). In earlier studies by others on perf-/-x*gld* mice, reduced pathology due to reduced vascular leakage and inflammation was observed (Braun et al., 1996; Martin et al., 1998; Rafi et al., 1998; Simpson et al., 1998; Kehren et al., 1999; Kreuwel et al., 1999; Perry et al., 1999). Accordingly, vascular leakage mediated by cytolytic NK/Tc cells, at the level of the BBB, in wt mice may explain why they have a greater susceptibility to infection with MVE than double-deficient mice.

The reduced susceptibility to MVE infection of mice that are only deficient in either the granule exocytosis- or the Fas-mediated effector functions (perf-/-, gzmAxB-/-, perfxgzmAxB-/- and *gld* mice, see Chapter 6) relative to wt mice I found in my experiments, supports the notion that cytolytic lymphocytes are mainly deleterious in this disease. However, these mice had slightly increased virus titers in the spleen and the brain. Even though the virus was detected more often, at an earlier time and with a delayed clearance in the tissues of the mutant mice, this did not increase their mortality but, on the contrary, reduced and delayed it. This suggests that the presence of the virus is not the only factor that contributes to morbidity and mortality. Thus, cell-mediated cytotoxicity is important for viral clearance, but it could also contribute to virus-triggered immunopathology.

The significantly increased resistance to MVE infection of the $ß_2$-M-/- mice compared to wt mice found in my experiments is further support by the detrimental role that Tc cells can play in this mouse model during an encephalitic virus infection. However, the virus load I found in the peripheral tissues of $ß_2$-M-/- mice was not very

different from that in wt mice. This finding is consistent with experiments on Sindbis virus (SV) infections by Kimura and Griffin (2000), who found that there was no difference in the amount of virus that was present in the tissues of wt C57Bl/6 and $ß_2$M-/- animals. Thus, experiments with both MVE and SV infections of $ß_2$M-/- mice suggest that $CD8^+$ T cells do not significantly reduce the virus load of these infections.

The Ab response I found in $ß_2$M-/- mice was intact because functional helper $CD4^+$ T cells and B cells were present. However, the increased resistance I found in the $ß_2$M-/- mice cannot be attributed to the antibody response. I found that the Ab titers in $ß_2$M-/- mice, after infection with MVE, were similar to those in wt mice. This was probably due to a similar level of antigen exposure, as there was no significant difference in MVE titers in tissues of infected $ß_2$M-/- and wt mice. In other studies, $ß_2$M-/- and wt mice also showed similar levels of Ab production after infection with vaccinia (for IgM) or vascular stomatitis (for IgM and IgG) viruses (Spriggs et al., 1992).

I obtained further evidence that Tc cells are not protective against MVE from passive transfer experiments that showed that transfer of MVE-immune T cells ($CD4^+$ and $CD8^+$) to wt mice infected with MVE, increased rather than reduced mortality. These preliminary results are in accordance with the results by Murali-Krishna et al. (1996), who injected JEV-primed T cells peripherally and found that they did not confer protection against JEV in mice.

On the other hand, I found that passive transfer of MVE-immune B cells provided complete protection from infection with MVE. This highlights the importance of Ab in the protection of mice from infection with MVE, and is consistent with recent work by Diamond et al. (2003b), which showed that B-cell-deficient mice uniformly succumb to WNV infection. Many other studies on the humoral response to flaviviruses

have also found that humoral immunity is essential for host survival. For example, the function of IgM in flaviviral infection is well characterized. The induction of a specific, neutralizing IgM response early in the course of WNV infection, limits viremia and dissemination of the virus into the CNS, and protects against lethal infection before the development of an adaptive IgG response (Diamond et al., 2003c). Low levels of IgM against JEV are a risk factor in JEV infection of humans, and the magnitude of anti-JEV IgM is considered a major prognostic indicator of disease outcome (Libraty et al., 2002). The role of immune IgG in protection has also been studied extensively in mouse models of flaviviral infections (Heinz et al., 1983; Mathews and Roehrig, 1984; Schlesinger et al., 1986; Kimura-Kuroda and Yasui, 1988; Kaufman et al., 1989; Mason et al., 1989; Schlesinger and Chapman, 1995; Kreil and Eibl, 1997; Broom et al., 2000; Roehrig et al., 2001; Ben-Nathan et al., 2003; Diamond et al., 2003b).

My studies with mice immunodeficient in T and B effector cells (RAG-1-/- mice) show that these mice had a significantly higher mortality compared to the wt mice. This demonstrates the importance of the adaptive immune response in the protection from MVE infection. The RAG-1-/- mice's increased mortality could be explained by their inability to have an antibody response, because they do not have B cells. However, compared to the time of death of the wt mice, the mortality of the RAG-1-/- mice, after iv inoculation of a low MVE dose, was significantly delayed. This observed delay in the onset of clinical signs of encephalitis and in the time to death of the RAG-1-/- mice was possibly due to the lack of the deleterious effect of the T cells. Cytotoxic effector mechanisms may be involved in the events that lead to neuroinvasion and in the pathology caused by the inflammation response in the brain. However, in similar work that studied infection with WNV this delay in time to death did not occur in RAG-1-/- (Halevy et al., 1994) and SCID mice (Diamond et al., 2003b). On the

contrary, in the both quoted research papers, the mortality of the immunodeficient mice was earlier than that of the wt mice. This difference in the pathogenesis of MVE and WNV is more likely due to the fact, alluded to earlier, that the Tc-cell response is important in the protection from WNV infection (Shrestha et al., 2003; Wang et al., 2003b) but not in the control of infection with MVE. The fact that the detection of MVE virus in extraneural and brain tissues of the RAG-1-/- mice in my experiments was also delayed (although with increased virus titers) is consistent with their delayed, but increased, mortality.

My histopathology results in organs of wt mice show that, even though there is mostly a correlation between inflammatory and necrotic foci and the level of MVE infection, it is difficult to establish whether brain injury is caused by MVE infection or by the resultant immune response of the host (e.g. infiltrating cells) using virological or histopathological criteria. My histology results from brain samples of MVE-infected $ß_2$-M-/- and RAG-1-/- mice, which showed a reduction in cell infiltration, indicate that there was a reduced inflammation. This may simply be due to the lack of $CD8^+$ T cells in $ß_2$-M-/- mice, and to the lack of T and B cells in RAG-1-/- mice. Tc and NK cell infiltration has been found in brains of WNV-infected wt mice (Liu et al., 1989; Wang et al., 2003b). RAG and $ß_2$-M-/- mice (similar to SCID mice) harbor functional macrophages and NK cells, which could well infiltrate the brains of MVE-infected mice, but do not seem to do so. Similarly, in studies made with the encephalitic flavivirus Montana Myotis leukoencephalitis, Charlier et al. (2002) found that there was little or no infiltration of macrophages or NK cells in the brains of infected SCID mice. In my study, the reduced cell infiltration in $ß_2$-M-/- mice may have accounted for the reduced and delayed mortality, and for the delayed neuroinvasion of RAG-1-/- mice after they had been infected with low to medium doses (0.1 to 10^5 PFU). However,

massive virus replication did occur in the brain after inoculation with 10^8 PFU in both immunodeficient strains of mice (similarly to wt mice), although the ATD was delayed.

In summary, the results from my *in vivo* study of the role of MVE-immune T cells and their specific cytotoxic mechanisms have confirmed the deleterious role of Tc cells. Lack or deficiency in these cells seem to be responsible for the delay in mortality of RAG-1-/- mice, for the delay and reduction in mortality of $ß_2$-M-/- mice, for the increase in resistance of mice that are deficient in either the degranulation (perforin/granzymes) or Fas pathway of cytotoxicity, and for the increase in resistance of mice that are deficient in both the degranulation and the Fas pathway of cytotoxicity (perf-/-x*gld*). These results have been fundamental to further research (presently continued by our JCSMR research group) on the local effect of Tc cells in the BBB and in the brain of flavivirus-infected mice, and could help to elucidate at what point Tc cells contribute to flavivirus pathogenesis.

The questions 'Does the increase in resistance in mice with a deficiency in cytolytic functions, which occurs after infection with MVE, also occur after infection with other encephalitic flaviviruses?', and 'Does this resistance occur in humans after infection with MVE?' remain open. It would certainly be of interest to test if this increased resistance applies to JEV, the most closely related encephalitogenic flavivirus to MVE, and the most important causative agent worldwide of viral encephalitis in humans.

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Mayan code for 'xekik' or yellow fever, circa 1480–1485. First written record known for a flaviviral disease (Bustamante, 1986).

'Can ahau u buluc it Katun cu xocol Chichen Itza uhe Katun ulom Huck, ulom yaxum ulom ulom (sic) ah Kante nal, *ulom xekik* tu can ua, ulom Kukulcan tu pach at Itgach tu canten u than atun vale'

Translation: In the Katun four auhau around a Katun, counted towards the well of Chichen Itzá in the place of the Katun rock, Kuk, Yaxun, Kantenal, Kukulcan (Quetzalcoatl), the vomit of blood will arrive for its fourth time.

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Appendix

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Lack of both Fas Ligand and Perforin Protects from Flavivirus-Mediated Encephalitis in Mice

Rosa M. Licon Luna,[1] Eva Lee,[1] Arno Müllbacher,[1] Robert V. Blanden,[1] Rod Langman,[2] and Mario Lobigs[1]*

Division of Immunology and Cell Biology, John Curtin School of Medical Research, The Australian National University, Canberra, Australian Capital Territory 2601, Australia,[1] and Conceptual Immunology Group, The Salk Institute, La Jolla, California[2]

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The mechanism by which encephalitic flaviviruses enter the brain to inflict a life-threatening encephalomyelitis in a small percentage of infected individuals is obscure. We investigated this issue in a mouse model for flavivirus encephalitis in which the virus was administered to 6-week-old animals by the intravenous route, analogous to the portal of entry in natural infections, using a virus dose in the range experienced following the bite of an infectious mosquito. In this model, infection with 0.1 to 10^5 PFU of virus gave mortality in ~50% of animals despite low or undetectable virus growth in extraneural tissues. We show that the cytolytic effector functions play a crucial role in invasion of the encephalitic flavivirus into the brain. Mice deficient in either the granule exocytosis- or Fas-mediated pathway of cytotoxicity showed delayed and reduced mortality. Mice deficient in both cytotoxic effector functions were resistant to a low-dose peripheral infection with the neurotropic virus.

Murray Valley encephalitis virus (MVE) is an Australian member of the encephalitic flaviviruses grouped into the Japanese encephalitis virus (JEV) serocomplex, which also includes West Nile virus (WNV) and St. Louis encephalitis virus (7, 37). The viruses are transmitted by mosquitoes to their normal vertebrate hosts (predominantly birds) and can cause incidental epidemic outbreaks of disease in humans. Most infections of humans with flaviviruses of the JEV serocomplex are subclinical, the estimated ratio of inapparent to apparent infection being between 200:1 and 1,000:1; however, the case fatality rate can be high (up to 50%), and life-long neuropsychological sequelae frequently occur among survivors of flaviviral encephalitis (for reviews, see references 33 and 37). Thus, an improved understanding of the pathogenesis of flaviviruses in animal models could significantly improve human health.

Mice provide an excellent animal model for flaviviral encephalitis in humans. Members of the JEV serocomplex are neurotropic in mice. When directly inoculated into the brain, they grow to high titers, causing a mostly fatal encephalomyelitis. The disease outcome following extraneural inoculation of the encephalitic flaviviruses into mice is strongly host factor dependent (for a review, see reference 35). For example, age of the animals is particularly important (11, 31, 32); mice up to 3 to 4 weeks of age are highly susceptible to a low-dose virus inoculum, whereas in older animals the peripheral injection of these flaviviruses often fails to result in morbidity or mortality over a wide dose range.

One disadvantage of the mouse model is the erratic course of infection in older mice, which requires a larger number of animals to make a reliable determination of the 50% lethal dose. A number of factors could cause the age-dependent differences in the susceptibility of mice to flavivirus infection; these include, but not exclusively, (i) higher peripheral virus yield and viremia in younger animals (32), (ii) an age-dependent difference in the permeability of the blood-brain barrier, (iii) a stronger tropism of the viruses for developing neurons (47, 48), and (iv) absence, delay, or dysfunction of innate and/or adaptive immune responses in the younger animals (52, 55, 63).

The key to controlling viremia is humoral immunity. Passive transfer of antibodies and vaccination-induced humoral immune responses can protect against lethal flavivirus challenge, including infection via the intracerebral (i.c.) route (4, 12, 22, 56). However, the kinetics of induction of humoral immunity in a primary flavivirus infection may not be sufficiently rapid to prevent neuroinvasion following peripheral virus growth and, in turn, fatal encephalitis. On the other hand, cellular antiviral immune responses are raised with a kinetics consistent with the requirement for rapid viral elimination (10, 20, 21). For example, primary WNV-immune cytotoxic T (Tc) cells are apparent at 5 days postinfection (p.i.), and the response peaks on day 7. Virus-immune Tc cells and natural killer (NK) cells have also been isolated from the brains of WNV-infected mice (26). However, it is still unclear whether these virus-induced cytolytic lymphocytes (NK/Tc cells) are necessary for the recovery from encephalitic flavivirus infection or whether they induce immunopathology, as observed in other viral models (5, 8, 38). Their role in recovery may depend on whether their function, when directed against virus-infected cells in the periphery, can prevent virus spread into the central nervous system (CNS).

Following neuroinvasion, encephalitic flaviviruses efficiently infect large numbers of neurons, and this process is accompanied by an inflammatory reaction (13, 15). The relative contributions to neuronal destruction and mortality of flaviviral

* Corresponding author. Mailing address: Division of Immunology and Cell Biology, John Curtin School of Medical Research, The Australian National University, P.O. Box 334, Canberra, A.C.T. 2601, Australia. Phone: (61)-2-6125 4048. Fax: (61)-2-6125 2595. E-mail: Mario.Lobigs@anu.edu.au.

infection of neurons, per se, and of the induced immunopathological events remain ambiguous (1, 24, 46).

Perforin-dependent granule exocytosis- and Fas-mediated cytotoxicity are the two major pathways causing target cell damage by cytolytic lymphocytes (14, 19). The availability of mouse strains defective in these cytotoxic effector pathways as a consequence of gene knockout or null mutations allows evaluation of the role of these two cytotoxic pathways in viral pathogenesis. Here we have applied this approach to a mouse model of flaviviral encephalomyelitis and revealed an unexpected Tc/NK-dependent pathogenicity component.

MATERIALS AND METHODS

Viruses and cells. The MVE prototype strain MVE-1-51 (9) was used. It has been passaged 15 times in suckling mouse brain. Working stocks were prepared from either 10% suckling mouse brain homogenates in Hanks' balanced salt solution (HBSS) containing 20 mM HEPES buffer (pH 8.0) and 0.2% bovine serum albumin (HBSS-BSA) or from infected *Aedes albopictus* C6/36 cell culture supernatant buffered to pH 8.0. Virus was stored in single-use aliquots at −70°C. Titers of working stocks ranged from 5×10^7 to 2×10^9 Vero cell PFU/ml.

Vero cells (African green monkey kidney; obtained from the American Type Culture Collection), and the methylcholanthrene-induced fibrosarcoma cells 2R (*H-2K*^{k}*D*^{b}) and 5R (*H-2K*^{b}*D*^{d}) and L929 cells (*H-2*^{k}) were maintained in Eagle's minimal essential medium (EMEM) plus nonessential amino acids and 5% fetal calf serum (FCS). C6/36 cells were grown in EMEM plus nonessential amino acids and 8% FCS at 28°C.

Animals. C57BL/6 (B6) mice, syngeneic gene knockout mice defective in perforin (*perf*$^{-/-}$) (17), granzymes A and B (*gzmAxB*$^{-/-}$) (58), or granzymes A and B plus perforin (*perf* × *gzmAxB*$^{-/-}$) (42), and syngeneic mutant mice with defects in the Fas receptor (*lpr*) (54) or Fas ligand (*gld*) (60) were bred under specific-pathogen-free conditions and supplied by the Animal Breeding Facilities at the John Curtin School of Medical Research, Canberra. Mice doubly deficient in the perforin and Fas pathways of cytotoxicity (*perf*$^{-/-}$ × *gld*) were generated by back-crossing breeders heterozygous for perforin and homozygous for the Fas ligand mutation (*perf*$^{+/-}$ × *gld*). This protocol was required because female *perf*$^{-/-}$ × *gld* mice are infertile (also reported in references 18 and 59). Weanling mice obtained from this mating were screened for homozygosity of the perforin knockout mutation and kept for experimentation. The screening was done by PCR on genomic DNA obtained by tail biopsies using a protocol described previously (58).

Plaque assay. Virus was titrated by plaque formation on ≈80% confluent monolayers of Vero cells (≈3×10^5 cells/well) in six-well plastic tissue culture plates (Linbro Scientific Inc., Hamden, Conn.). Samples to be assayed were serially diluted in HBSS-BSA on ice, and monolayers were inoculated in duplicate with 0.1-ml aliquots of the diluted virus. Adsorption was for 1 h at 37°C in an atmosphere of 5% CO_2 and 95% air with occasional shaking. An agar overlay medium (EMEM plus nonessential amino acids containing 1% Bacto-agar [Difco, Detroit, Mich.], 2% FCS, penicillin [30 μg/ml; Sigma, St. Louis, Mo.], streptomycin sulfate [50 μg/ml; Sigma], neomycin sulfate [50 μg/ml; Sigma], and amphotericin B [Fungizone; 0.25 μg/ml; Life Technologies, Rockville, Md.]; 4 ml/well) was added to the monolayer, and the cells were incubated for 72 to 96 h at 37°C. The monolayers were stained by the addition of 1.5 ml of 0.03% neutral red (BDH Chemicals, Poole, England) in HBSS, and plaques were counted 10 to 16 h later, following removal of the stain and the overlay.

For virus determination in infected mouse tissues, animals were sacrificed at a given time p.i., and tissues were aseptically removed, snap-frozen in liquid nitrogen or dry ice, and stored at −70°C. The 10% (wt/vol) tissue suspensions in ice-cold HBSS-BSA were homogenized and clarified by centrifugation (18,000 × *g* for 5 min at 4°C), and supernatants were stored in aliquots at −70°C prior to plaque titration. The limits of detection of virus in tissues and serum of infected mice by plaque titration were 10^3 PFU/g and 10^2 PFU/ml, respectively.

ELISA. MVE antigen-coated 96-well microtiter plates were prepared as previously described (6). Serial twofold dilutions (50 μl/well) of antisera in phosphate-buffered saline (PBS) containing 1% BSA and 0.05% Tween 20 (Sigma; PBS/BSA/Tween) were added to the plates and incubated for 2 h at room temperature. The plates were washed four times with PBS containing 0.05% Tween 20 before the addition of 50-μl/well horseradish peroxidase-conjugated goat anti-mouse immunoglobulin (Ig; Dako, Carpinteria, Calif.) diluted 1:1,000 in PBS/BSA/Tween for 1 h at room temperature. The wells were washed as above, 50 μl of the peroxidase substrate 2.2′-azino-di[3-ethyl-benzthiazoline sulfonate] (ABTS; Kirkegaard & Perry Laboratories, Gaithersburg, Md.) was added per well, and the plates were kept at room temperature for ~20 min to allow color development. The enzymatic reaction was stopped by the addition of 1% sodium dodecyl sulfate, and plates were read at 405 nm with a Molecular Dynamics microplate reader.

Optical density (OD) cutoff values were established as the mean OD of eight negative control wells containing sera from naïve mice plus 3 standard deviations (SD). OD values were considered positive if they were equal to or greater than the OD cutoff. Endpoint titers were calculated as the reciprocal of the last dilution that was positive, and the antibody titers are given as $\log_{10}$ values.

Chromium release assay. The generation of secondary in vitro MVE-immune effector Tc cells, target cell infections, and ^{51}Cr release assay were performed as described previously (27, 51). Briefly, B6 mice were immunized intraperitoneally with 5×10^6 PFU of MVE, spleens were harvested at 7 days p.i., and single-cell suspensions were prepared. One-fifth of the spleen cell suspension was infected with MVE at a multiplicity of 5 PFU/cell for 1 h, washed, and cultured with the rest of the splenocytes for 5 days. 2R and 5R target cells were infected with MVE at a multiplicity of 50 PFU/cell for 16 h prior to ^{51}Cr labeling for 1 h and incubation with titrated numbers of effector cells for 6 h. All samples were triplicates, the standard error of the mean (SEM) was always <3%, and spontaneous release was between 10 and 20%.

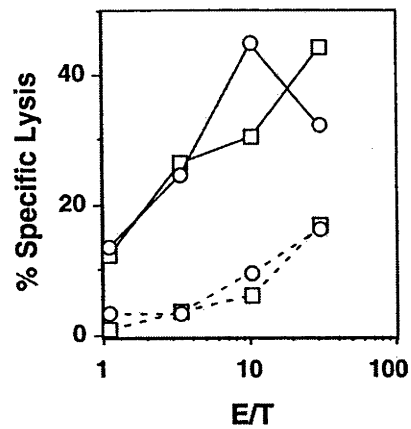

FIG. 1. MVE infection of B6 mice elicits H-2^b-restricted antiviral Tc cell responses. Lysis of ^{51}Cr-labeled 2R (□) and 5R (○) cells infected with MVE (solid line) or left uninfected (dashed line) by secondary, in vitro-stimulated MVE-immune Tc cells from B6 mice was measured. Representative data from one of four experiments are shown. The SEM was always <3%. E/T, effector-to-target cell ratio.

RESULTS

In vitro cytotoxicity of splenocytes from *H-2*^{b} mice against MVE-infected target cells. We investigated the role of the cytolytic effector functions of NK and Tc cells in a mouse model of flaviviral encephalitis. We have previously reported on the H-2^k- and H-2^d-restricted Tc cell responses against MVE (27, 29, 51). To verify that MVE-specific Tc cell responses are also induced in *H-2*^{b} (B6) mice, secondary, in vitro-stimulated, MVE-immune splenocytes were tested against MVE-infected and uninfected 2R (*K*^{k}, *D*^{b}) and 5R (*K*^{b}, *D*^{d}) target cells in ^{51}Cr release assays. Figure 1 shows that D^b- and K^b-restricted anti-MVE Tc cell responses are elicited in MVE-infected B6 mice. No MVE-specific killing of H-2-mismatched MVE-infected target cells (L929, *H-2*^{k}) by the MVE-immune effector cells was observed (data not shown), showing that the D^b- and K^b-restricted lysis was Tc cell mediated.

Mortality caused by MVE in 6-week-old B6 mice. Inoculation by the intravenous (i.v.) route of 6-week-old B6 mice with MVE characteristically failed to produce a dose-dependent

TABLE 1. Susceptibility of B6 mice, wild type or defective in granule exocytosis- or Fas-mediated cytotoxicity, to i.v. infection with MVE

Dose (PFU)	Mouse strain	No./ group	% Mortality[a]	AST ± SEM[a]
0.1	Wild type	27	59	10.3 ± 0.6
	perf$^{-/-}$	12	33	11.0 ± 0.7
	gzmA×*B*$^{-/-}$	18	28[1]	11.6 ± 0.6
	perf × *gzmA*×*B*$^{-/-}$	6	33	11.0 ± 1.0
	gld	10	40	12.3 ± 1.5
	lpr	6	33	14.0 ± 0[1]
10^2	Wild type	70	46	11.6 ± 0.4
	perf$^{-/-}$	15	20[1]	12.3 ± 2.2
	gzmA×*B*$^{-/-}$	20	25	10.8 ± 1.5
	perf × *gzmA*×*B*$^{-/-}$	19	32	12.5 ± 0.3
	gld	11	27	11.7 ± 0.7
	lpr	11	55	11.3 ± 1.1
10^3	Wild type	9	56	12.0 ± 0.8
	perf$^{-/-}$	16	38	12.8 ± 0.7
10^5	Wild type	20	40	10.9 ± 0.8
	perf$^{-/-}$	12	33	13.3 ± 1.0[1]
	gld	14	36	12.8 ± 1.0
	lpr	10	80[1]	10.8 ± 0.6
10^8	Wild type	19	100	5.5 ± 0.2
	perf$^{-/-}$	10	100	6.6 ± 0.2[3]
	gzmA×*B*$^{-/-}$	6	100	6.2 ± 0.4
	perf × *gzmA*×*B*$^{-/-}$	5	100	9.4 ± 1.4[3]
	gld	10	100	6.6 ± 0.3[2]
	lpr	5	100	5.8 ± 0.2

[a] Statistical significance relative to groups of wild-type mice is indicated by superscript numbers as follows: 1, $P \leq 0.1$; 2, $P \leq 0.05$; and 3, $P \leq 0.01$. Differences in mortality were assessed using Fisher's exact test, and differences in AST were assessed using the Mann-Whitney test.

increase in mortality. A high virus dose ($\geq 10^8$ Vero cell PFU) consistently resulted in 100% death, whereas virus doses in the range from 0.1 to 10^5 PFU killed ~50% of the animals (Table 1). This pattern, which we also observed for the encephalitic flavivirus WNV (unpublished data), was reflected in two distinct kinetics for the average survival time (AST). Mice injected with 10^8 PFU of MVE died at 5 to 6 days p.i., whereas mortality caused by the lower doses occurred from days 9 to 13 p.i. (Table 1). When 10^2 PFU of MVE was inoculated by the i.c. route into 6-week-old B6 mice, death uniformly occurred at days 5 to 7 p.i. (see Fig. 5D).

Accordingly, it appears that i.v. inoculation of 10^8 PFU results in early viral invasion of the CNS, with a disease outcome comparable to that following i.c. injection of the virus. The longer time to death in mice infected i.v. with the lower virus doses suggests that peripheral virus growth was required prior to the time that neuroinvasion took place. However, a 10^6-fold difference in the size of the initial i.v. inoculum did not result in any significant change in the AST. Animals that succumbed to high- or low-dose i.v. infections with MVE showed similar clinical signs at 1 to 2 days prior to death, which included wasting, ruffled fur, a hunched posture, and hind limb paralysis. These observations, in addition to comparable virus titers in the brains of moribund mice from groups infected i.v. with 10^8 PFU of MVE or the lower doses (see Fig. 3), suggested that in both cases viral encephalitis was the cause of death.

Role of perforin and granzymes in the pathogenesis of mice infected with MVE. Killing of virus-infected cells by cytolytic lymphocytes via the granular exocytosis mechanism involves at least two defined components of the secreted granules, perforin and the granzymes (39). To investigate whether granular exocytosis-mediated cytotoxicity by NK/Tc cells is important in recovery of mice from infection with an encephalitic flavivirus, the outcome of MVE infection was tested in knockout mice with defects in perforin (*perf*$^{-/-}$), granzymes A and B (*gzmAxB*$^{-/-}$), and perforin plus the two granzymes (*perf* × *gzmAxB*$^{-/-}$). Groups of B6 wild-type and knockout mice were inoculated i.v. with doses of MVE ranging from 0.1 to 10^8 PFU and observed for 3 weeks to record mortality and AST.

Table 1 shows that in the dose range of 0.1 to 10^5 PFU of MVE, mortality in the groups of *perf*$^{-/-}$, *gzmAxB*$^{-/-}$, and *perf* × *gzmAxB*$^{-/-}$ mice was always lower than that for the wild-type mice. When mortality data in this dose range for groups of *perf*$^{-/-}$ and *gzmAxB*$^{-/-}$ mice are summed, a statistically significant increase in resistance to MVE infection is found for the two strains relative to wild-type mice ($P = 0.022$ and 0.014, respectively; Fisher's exact test). Given the relatively small number of *perf* × *gzmAxB*$^{-/-}$ mice that were available for this study, the difference in additive mortality values for this knockout mouse (8 dead, 17 alive) and those for the wild-type (59 dead, 59 alive) was not statistically significant ($P = 0.125$). However, a significant increase in AST of *perf* × *gzmAxB*$^{-/-}$ (9.4 days) relative to wild-type mice (5.5 days) was noted ($P = 0.009$; Mann-Whitney test) in groups injected i.v. with 10^8 PFU of MVE (Table 1). This difference was less obvious for *perf*$^{-/-}$ and *gzmAxB*$^{-/-}$ mice, for which the AST was prolonged by ~1 day. The comparison of the ASTs for groups of mice inoculated with the lower doses of MVE (0.1 to 10^5 PFU) also showed a trend towards a slightly increased AST of mice deficient in the granular exocytosis pathway of cytotoxicity relative to the wild-type mice.

In summary, the data in Table 1 indicate that granule exocytosis-mediated cytotoxicity of NK/Tc cells has no protective value in MVE infection; in fact, it appears to accelerate the fatal outcome of viral growth in the CNS and increase mortality rates.

Role of Fas-mediated cytotoxicity in the pathogenesis of mice infected with MVE. The second major cytolytic mechanism of NK/Tc cells involves the engagement of Fas on the target cell by Fas ligand (FasL) (for a review, see reference 43). This mechanism is essential in immune regulation, demonstrated by a lymphoproliferative disease in mice with a loss-of-function mutation of the Fas gene (*lpr*) or a point mutation in the FasL gene (*gld*) (44, 45). The Fas-mediated death pathway has been implicated in the control of some viral infections (61, 62). In the absence of functional FasL expression (*gld* mutant mouse), the mortality inflicted in the i.v. dose range of 0.1 to 10^5 PFU of MVE was always less than that found for wild-type B6 mice (Table 1). In addition, the AST was significantly increased in *gld* mice relative to wild-type mice when 10^8 PFU was injected i.v. ($P = 0.014$). The Fas defect in *lpr* mice showed no consistent trend in the outcome of infection with MVE (Table 1). However, it is known that the *lpr* mutation is leaky and that small amounts of functional Fas are expressed in the mutant mouse (3).

Virus growth in tissues of B6 wild-type mice and mutant mice with defects in the granule exocytosis- or Fas-mediated cytotoxic effector mechanism. Tissues (spleen, muscle, lymph nodes, and liver) from 6-week-old B6 wild-type, *perf*$^{-/-}$, *gld*,

TABLE 2. MVE-specific antibody responses in C57B1/6 wt mice and in mutant mice deficient in granule exocytosis and/or Fas pathways of cytotoxicity

Expt no.	Virus dose (PFU)	Mouse strain	Time (days p.i.)	No./group	% Seroconversion	Mean antibody titer,[a] $\log_{10}$ (range)
1	0.1	Wild type	10	6	100	3.7 (2.6–4.1)
		perf$^{-/-}$		3	100	4.0 (3.8–4.1)
		gzmAxB$^{-/-}$		4	100	4.2 (4.1–4.4)
		gld		3	100	3.7 (2.6–4.4)
		Wild type	21–25	4	100	4.3 (3.8–4.7)
		perf$^{-/-}$		3	100	4.6 (4.1–5.0)
		gld		1	100	5.0
	10^2	Wild type	21–32	11	100	4.0 (2.9–4.7)
		perf$^{-/-}$		4	100	4.3 (4.1–4.4)
		gzmAxB		5	100	3.8 (3.5–4.1)
		perf × *gzmA* × *B*$^{-/-}$		8	100	4.0 (3.8–4.1)
		gld		8	100	4.5 (3.5–5.0)[b]
		lpr		4	100	4.3 (3.8–4.7)
2	10^5	Wild type	5	6	100	2.5 (2.0–2.9)
		perf$^{-/-}$ × *gld*		3	100	2.7 (2.6–2.9)
		Wild type	10	7	100	3.4 (3.2–3.5)
		perf$^{-/-}$ × *gld*		4	100	4.0 (3.8–4.4)[b]
		Wild type	15	7	100	3.7 (3.5–3.8)
		perf$^{-/-}$ × *gld*		4	100	4.1 (3.5–4.7)
		Wild type	21	7	100	3.8 (3.5–4.1)
		perf$^{-/-}$ × *gld*		4	100	4.5 (4.1–4.7)[b]
		gld		3	100	4.5 (4.4–4.7)[b]
		perf$^{-/-}$		3	100	4.0 (3.8–4.1)

[a] MVE-specific antibody titers were determined by ELISA, and endpoints were calculated as described in Materials and Methods.
[b] Statistically significant difference ($P \leq 0.05$; Mann-Whitney test) relative to groups of wild-type mice.

and *lpr* mice infected i.v. with 10^8 or 10^5 PFU of MVE were tested at 1- and 2-day intervals, respectively, for the presence of virus or viral RNA by plaque titration and reverse transcription (RT)-PCR. Consistent with earlier studies (32), we found that MVE grows poorly in extraneural tissues of mice of this age. When inoculated i.v. with 10^5 PFU of MVE, barely detectable virus titers were found in the spleen at 4 days p.i., and virus was cleared by 6 days p.i.; virus was not detectable in lymph nodes, muscle, liver, or blood (data not shown), although all the mice had seroconverted (Table 2). Inoculation i.v. of 10^8 PFU resulted in virus detection in the spleen, muscle (Fig. 2), and lymph nodes, but not the liver (data not shown).

No consistent difference in growth kinetics or magnitude of virus titers was apparent between wild-type mice and mice defective in NK/Tc cell cytotoxic effector mechanisms. Virus titers in the spleen were highest on day 2 and lowest on day 4 p.i.. This pattern was also found for virus titers in lymph nodes (data not shown). MVE appeared to grow in muscle from day 2 to day 4 p.i. but was not detectable in all animals. No virus was detectable in the blood ($<10^2$ PFU/ml) 2 days after i.v. injection of 10^8 PFU of MVE. The apparent inability of extraneural tissues to support efficient growth of MVE was confirmed by RT-PCR on samples from liver, spleen, muscle, kidney, ovaries, lymph nodes, and blood obtained from infected mice at 2, 4, 6, and 8 days p.i. (unpublished data). The sensitivity of detection of viral RNA by the RT-PCR method was estimated at $\sim 10^4$ templates/reaction.

Unfortunately, these data do not permit us to say whether (i) NK/Tc cell-mediated cytotoxicity contributes to the control of virus infection in these tissues or (ii) the magnitude of virus titers in extraneural tissues is a decisive factor that determines the incidence of neuroinvasion and thus can account for the variability between individuals in the susceptibility to infection with MVE.

Kinetics of neuroinvasion and growth of MVE in the brain of wild-type mice and mutant mice defective in granule exocytosis- or Fas-mediated cytotoxicity. Groups of 6-week-old mice were infected i.v. with either 10^8 or 10^2 PFU of MVE. Individuals were sacrificed at 2, 3, and 4 days after 10^8 PFU and 6 and 8 days after 10^2 PFU inoculations, and virus titers in the brain were determined. The dose of 10^8 PFU i.v. gave detectable virus titers in the brains of most animals at 2 days p.i. (ranging from 10^3 to 10^6 PFU/g), which increased to titers of 10^7 to 10^9 PFU on day 4 p.i. in the *perf*$^{-/-}$, *gld*, and *lpr* mice and in some of the wild-type mice (Fig. 3). The mean titers of MVE in the brains of the four strains of mice were comparable, but wild-type mice showed much greater variability than the other groups on day 3 and day 4 p.i..

After inoculation i.v. of 10^2 PFU of MVE, low titers of virus were found on day 6 p.i. in brains of a few mice in the *gld*, *lpr*, and *perf* × *gzmAxB*$^{-/-}$ but not the wild-type and *perf*$^{-/-}$ groups (Fig. 3). By day 8 p.i., virus was found in six of eight wild-type mice, and titers ranged from 10^3 to 10^8 PFU/g. Members of the groups of *perf*$^{-/-}$, *gld*, and *perf* × *gzmAxB*$^{-/-}$ mice had high brain titers (10^7 to 10^9 PFU/g) on day 8 p.i., but overall the majority of mice defective in granule exocytosis- or

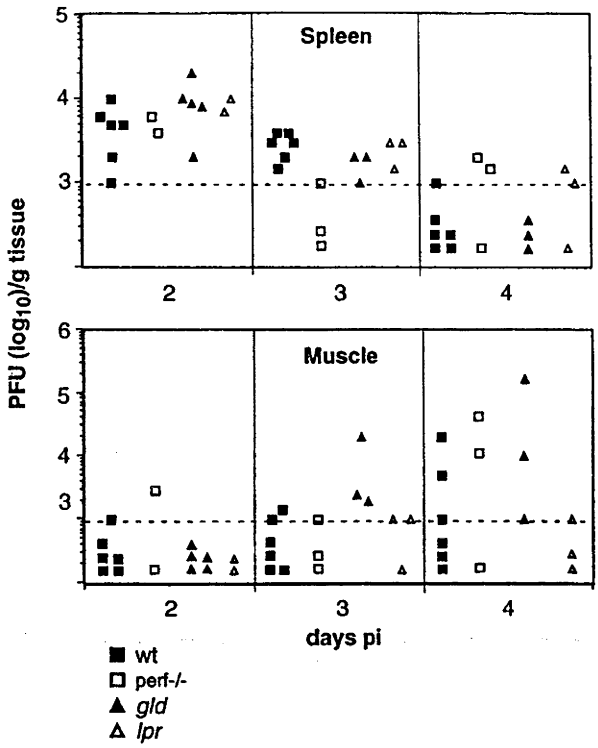

FIG. 2. Growth of MVE in spleen and muscle of B6 wild-type (wt) mice and mice deficient in granule exocytosis- or Fas-mediated cytotoxicity. Mice (6 weeks old) were infected i.v. with 10^8 PFU of MVE. At the indicated times, animals were sacrificed, and virus titers in tissues were determined by plaque titration. Each symbol represents an individual mouse. The lower limit of virus detection was 10^3 PFU/g tissue and is indicated by the dashed line.

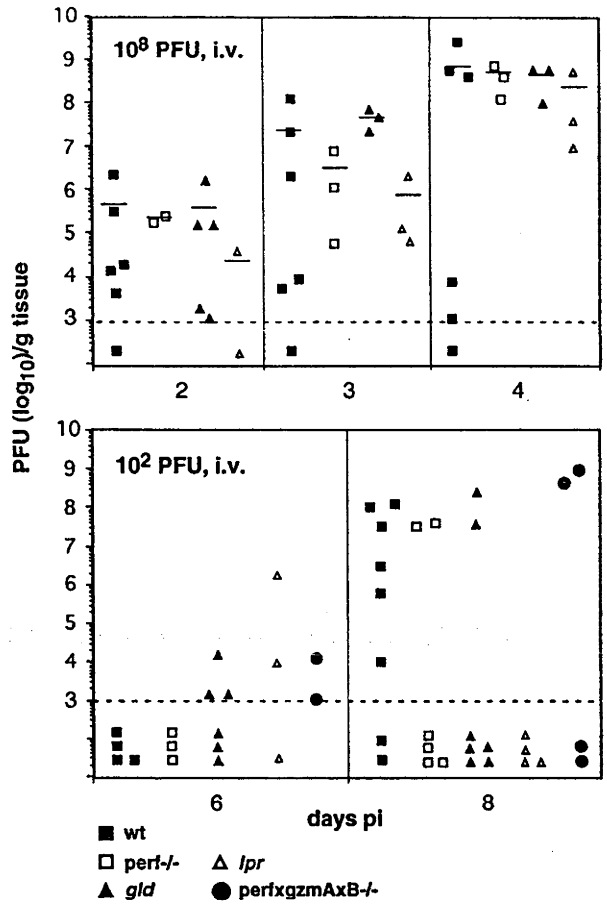

FIG. 3. Kinetics of neuroinvasion and growth of MVE in the brain of wild-type (wt) mice and mice defective in granule exocytosis- or Fas-mediated cytotoxicity. Groups of mice (6 weeks old) were infected i.v. with 10^2 or 10^8 PFU of MVE. At the indicated times, animals from each group were sacrificed, and virus titers in the brain were determined by plaque titration. Each symbol represents an individual mouse. The lower limit of detection of virus titers was 10^3 PFU/g of tissue and is indicated by the dashed line. To calculate mean titers (indicated by a horizontal line), values below the limit of detection were ignored.

Fas-mediated cytotoxicity had no detectable virus in the brain, consistent with the lower mortality rates in these mice relative to wild-type mice.

Resistance to infection with MVE of mice doubly deficient in granule exocytosis- and Fas-mediated cytotoxic pathways. The above results suggest that mice with functional defects in the granule exocytosis- or Fas-mediated pathway of cytotoxicity are less susceptible to lethal infection with MVE than wild-type mice. This implies that NK/Tc cell-mediated killing contributes to the pathogenesis of encephalitic flaviviruses in their vertebrate hosts. Given the possibility of redundancy between the two major pathways of cytotoxicity (49, 61, 62), the detrimental role of NK/Tc cells in encephalitic flaviviral disease may have been missed or underestimated in studies using mice defective in only one of the two pathways.

To investigate this possibility, mice defective in both cytotoxic effector mechanisms (*perf*$^{-/-}$ × *gld*) were generated. Consistent with earlier reports (18, 59), we found that these doubly deficient mice developed a severe autoimmune syndrome characterized by splenomegaly and lymphadenopathy, resulting in weight loss and death between the ages of 4 and 16 weeks (Fig. 4). About one-third of doubly deficient mice died between the ages of 6 and 9 weeks, but the clinical manifestations of MVE infection were distinguishable from signs of lymphoproliferative disease in these animals.

Groups of 6-week-old *perf*$^{-/-}$ × *gld* and wild-type mice were infected i.v. with 10^2 or 10^5 PFU of MVE, and morbidity and mortality were recorded daily for 21 days. All animals in a group of eight *perf*$^{-/-}$ × *gld* mice inoculated with 10^5 PFU survived, in contrast to 40% mortality in the wild-type group (Fig. 5B). Similarly, fatal viral encephalitis did not occur in the group of *perf*$^{-/-}$ × *gld* mice infected with 10^2 PFU (Fig. 5A). However, 4 of 12 doubly deficient mice in this group died without displaying the characteristic clinical signs of viral encephalitis, and MVE could not be recovered from the brains of cadavers within a period of 16 h after death had occurred. Since acute lymphadenopathy was seen in all cadavers and the mortality rate was consistent with that in the uninfected control group (Fig. 4), we conclude that death was most likely due to lymphoproliferative disease.

The susceptibility to i.v. infection with 10^2 PFU of MVE of 6-week-old *gld* mice heterozygous for the perforin knockout mutation (*perf*$^{+/-}$ × *gld*) was marginally lower than that of wild-type mice (33 and 45%, respectively; Fig. 5A) and com-

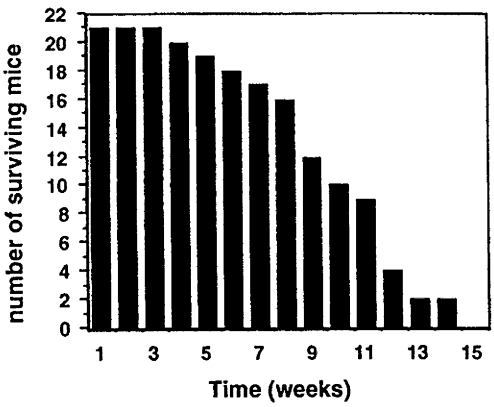

FIG. 4. Time to death of animals in a cohort of *perf*$^{-/-}$ × *gld* mice.

parable to that of *gld* mice (Table 1). This is indicative of a lack of gene dose effect in the contribution of perforin on the *gld* background to the pathogenesis of MVE.

Infection of *perf*$^{-/-}$ × *gld* mice with 10^8 PFU of MVE by the i.v. route gave 100% mortality in 6-week-old animals and 75% mortality in 10-week-old mice (Fig. 5C). These values were comparable to those for the wild-type mice. However, the AST was longer in the doubly deficient animals relative to wild-type mice (7.3 and 5.6 days, respectively, for 6-week-old mice and 9.7 and 7.0 days, respectively, for 10-week-old mice). Intracerebral injection of 10^2 PFU of MVE in 6-week-old wild-type and *perf*$^{-/-}$ × *gld* mice resulted in 100% mortality, with comparable kinetics to death in both mouse strains (Fig. 5D).

To further substantiate that virus growth in the CNS of the doubly deficient mice was not suppressed, 6-week-old *perf*$^{-/-}$ × *gld* mice were infected i.v. with 10^8 PFU of MVE, and brain titers were determined on days 3 and 4 p.i. No difference was found between the MVE titers in the brain of *perf*$^{-/-}$ × *gld* mice on day 3 p.i. (3.5×10^6 to 2.5×10^8 PFU/g) and day 4 p.i. (2.8×10^9 to 4.5×10^9 PFU/g) and those found in wild-type mice (Fig. 3). Thus, in *perf*$^{-/-}$ × *gld* mice, neuroinvasion by MVE occurred following high-dose i.v. inoculation, and virus growth in the brain was not inhibited.

MVE-specific antibody responses in B6 wild-type mice and mutant mice deficient in granule exocytosis and/or Fas pathway of cytotoxicity. Single vaccination of B6 mice with 10^6 PFU of UV-inactivated MVE does not induce MVE-specific antibodies detectable by ELISA (unpublished data). Seroconversion of mice following inoculation of smaller doses of live virus (0.1 to 10^5 PFU) would therefore be consistent with a productive infection. To verify that i.v. infections of mice deficient in the granule exocytosis- and/or Fas-mediated pathway of cytotoxicity with low doses of MVE resulted in virus growth in extraneural tissues, and to test whether they showed differences in the magnitude of the humoral immune response against MVE, antibody titers and incidence of seroconversion were determined.

The sera from all animals infected with MVE at 0.1 to 10^5 PFU were reactive against MVE antigen (Table 2). No dose-dependent difference was seen in the magnitude of the antibody response in wild-type mice. MVE-specific antibody titers in *perf*$^{-/-}$ × *gld* mice were four- to sevenfold higher than those in wild-type mice at 10 to 21 days p.i., although the difference in mortality between the two groups may have been a biasing factor, since only those mice that survived the virus infection until serum collection were tested. A significant increase in the magnitude of the humoral immune response against MVE was also seen in some groups of *gld* but not *perf*$^{-/-}$, *gzmAxB*$^{-/-}$, *perf* × *gzmAxB*$^{-/-}$, and *lpr* mice relative to wild-type mice (Table 2).

DISCUSSION

We have investigated the contribution of the cytotoxic effector functions of NK/Tc cells to recovery from and/or pathogenesis of encephalitic flavivirus infection using mutant mouse strains defective in the granule exocytosis and Fas or pathway of cytotoxicity. We first established parameters in immunologically mature mice, allowing the study of pathogenesis and control of infection with MVE, a member of the JEV serocomplex. Using 6-week-old B6 mice, we found that, depending on the virus dose used for i.v. inoculation, two kinetically distinct disease processes led to fatal encephalitis. A high dose of MVE given i.v. (10^8 PFU) gave pathogenesis comparable to that with i.c. injection of 10^2 PFU with respect to the first appearance of signs of encephalitis on day 4 p.i., the AST (5 to 6 days), and disease outcome (100% mortality). This pattern is consistent with the notion that sufficient virus from a high i.v. dose can enter the brain directly from blood to establish a lethal infection without the requirement for replication in extraneural tissues.

This rapid mode of invasion of the brain contrasted with the delayed kinetics and variable incidence of virus entry into the brain when lower i.v. doses of MVE, in the range of 0.1 to 10^5 PFU, were used. Virus first appeared in the brain between 6 and 8 days p.i., and mortality was observed between 9 and 13 days p.i. in ~50% of infected mice. This disease pattern suggests that the virus first replicated in extraneural tissues prior to entering the CNS and that invasion of the brain was a stochastic process unrelated to the dose of virus injected in the range of 0.1 to 10^5 PFU. Infection of mice with low doses of MVE rarely gave rise to detectable virus in the extraneural tissues tested and never produced a detectable secondary viremia, but all animals seroconverted, confirming productive virus infection.

The lack of any difference in AST over a 10^6 range of i.v. virus inocula indicates that the kinetics of neuroinvasion was surprisingly dose insensitive and that equalizing factors exist. We propose that the kinetics of induction of the antiflaviviral Tc cell response, which also displays a remarkably flat dose response at low to medium virus concentrations (20), is one such factor. In addition, the antiviral alpha/beta interferon response is most important in controlling flavivirus growth in extraneural tissues, given that adult alpha interferon receptor knockout mice rapidly succumb to an acute fulminant infection when inoculated with 10^2 PFU of MVE (unpublished data). The magnitude of the interferon response may inversely correlate with the virus dose inoculated.

The mechanism that allows encephalitic flaviviruses to breach the blood-brain barrier remains uncertain, but four candidate routes for CNS invasion have been canvassed (for

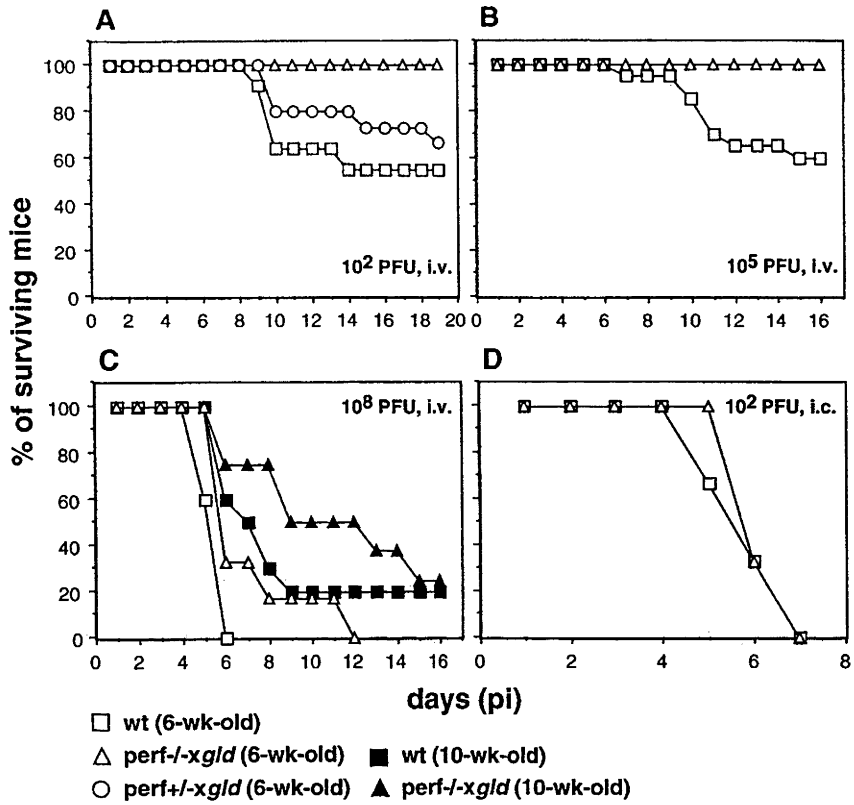

FIG. 5. Susceptibility of *perf*$^{-/-}$ × *gld* mice to infection with MVE. (A) Groups of 6-week-old wild-type (wt) (n = 11), *perf*$^{-/-}$ × *gld* doubly deficient (n = 8), and *perf*$^{+/-}$ × *gld* heterozygous (n = 15) mice were infected i.v. with 10^2 PFU of MVE. Mortality due to lymphoproliferative disease occurred in the group of doubly deficient mice on days 9 (n = 1) and 13 (n = 3) and was excluded from the total. (B) Groups of 6-week-old wild-type (n = 20) and *perf*$^{-/-}$ × *gld* (n = 8) mice were infected i.v. with 10^5 PFU of MVE. (C) Groups of 6-week-old and 10-week-old wild-type (n = 5 and 10, respectively) and *perf*$^{-/-}$ × *gld* (n = 6 and 8, respectively) mice were infected i.v. with 10^8 PFU of MVE. (D) Time to death of 6-week-old wild-type (n = 3) and *perf*$^{-/-}$ × *gld* (n = 3) mice infected i.c. with 10^2 PFU of MVE. In all experiments, morbidity and mortality were recorded daily, and surviving mice were monitored for 21 days.

reviews, see references 16 and 35): (i) by the neuronal route after infection of peripheral nerves; (ii) by infection of highly susceptible olfactory neurons, which are unprotected by the blood-brain barrier (36); (iii) by virus entry into vascular endothelial cells of capillaries in the brain, transcytosis, and release of virus into the brain parenchyma (25); and (iv) by diffusion of virus between capillary endothelial cells in individuals displaying leakiness of the blood-brain barrier due to factors unrelated or secondary to the viral infection (13, 23, 30). Antiviral immune responses could contribute to the fourth mechanism by inducing breakdown of the blood-brain barrier. Given the distinct modes of disease progression leading to fatal encephalitis that were observed when high (10^8 PFU) and lower (0.1 to 10^5 PFU) doses of MVE were inoculated i.v. and the concomitant differences in virus load in the vascular compartment, the possibility that encephalitic flaviviruses can breach the blood-brain barrier by a number of different mechanisms must be considered.

In addition to establishing conditions for investigating the role of the cytotoxic effector function of splenocytes in recovery or disease from MVE infections in a mouse model, we have confirmed that B6 mice mount H-2^b-restricted Tc cell responses against MVE. The role of NK cells in flavivirus immunity appears to be evaded due to the upregulation of major histocompatibility complex (MHC) class I by flavivirus infection and, as a consequence, the inhibition of NK cell-mediated infected target cell lysis (28, 34, 41).

The most striking finding in this study is the demonstration that cytotoxic effector pathways of NK/Tc cells contribute to accelerated and more severe pathogenesis of encephalitic flavivirus infection. Cytotoxic effector mechanisms may be involved at two stages in the disease process: first, in the events leading to neuroinvasion, and second, in the pathology due to the inflammatory response in the brain. Both the granule exocytosis- and the Fas-mediated pathways of cytotoxicity can contribute to disease progression with potentially compensatory function in mice when one of the two mechanisms is defective. Thus, contrary to the expectation based on the established importance of Tc and NK cells and their cytolytic mechanism in control and clearance of infection in other virus models (2, 17, 39, 40, 64), doubly deficient *perf*$^{-/-}$ × *gld* mice displayed a greatly increased resistance to i.v. infection with MVE at virus doses which gave ~50% mortality in wild-type mice. In support of the detrimental role of CD8$^+$ T cells in the recovery from encephalitic flavivirus infection, we also found a significantly reduced mortality of β_2-microglobulin knockout

mice, which lack $CD8^+$ T cells and expression of their restriction elements, in comparison to wild-type mice when infected with 10^2 PFU of MVE i.v. (data not shown).

The Fas pathway plays a critical role in lymphocyte homeostasis, which accounts for a severe autoimmune syndrome characterized by enlarged spleen and lymph nodes and lymphocyte infiltration in liver and kidney in Fas receptor (*lpr*)- and FasL (*gld*)-deficient mice (43). This disease is exacerbated in the absence of functional perforin, and, consistent with previous reports (18, 59), *perf*$^{-/-}$ × *gld* mice died at between 4 and 16 weeks of age. Mortality due to unchecked expansion of activated T cells in *perf*$^{-/-}$ × *gld* mice could be distinguished from that caused by MVE infection. Thus, we did not observe clinical signs typically associated with flaviviral encephalitis in any of the infected *perf*$^{-/-}$ × *gld* mice inoculated i.v. with 10^2 or 10^5 PFU of MVE. In addition, these doses did not result in virus titers detectable by plaque titration in the brains of mice that died during the virulence assays (most likely due to the lymphoproliferative syndrome) or that were sacrificed at 4 ($n = 2$), 6 ($n = 2$), and 8 ($n = 4$) days p.i. (data not shown). Accordingly, we conclude that in the absence of the two major cytotoxic pathways of NK/Tc cells, neuroinvasion by encephalitic flaviviruses is prevented. Presumably neuroinvasion by MVE in this model requires extraneural virus growth for several days following primary virus inoculation into the bloodstream, resembling natural infection by the bite of an infected arthropod.

What is the mechanism by which a deficiency in the two main cytotoxic effector functions prevents fatal flaviviral encephalitis? It is conceivable that cytotoxic cells disrupt the blood-brain barrier by killing infected endothelial cells lining the brain capillaries, allowing virus access into the brain parenchyma. *perf*$^{-/-}$ and *gld* mice display a marked decrease in vascular leakage resulting from endothelial cell injury (50), and the double deficiency in *perf*$^{-/-}$ × *gld* mice may entirely prevent capillary leakage due to the effector functions of cytotoxic lymphocytes. A gradation in the severity of vascular endothelium cytolysis in wild-type, *perf*$^{-/-}$, *gld*, and doubly deficient mice may account for the progressive reduction in mortality following i.v. low-dose MVE infection found in these mouse strains. Thus, the key stochastic factors allowing neuroinvasion in infections with low virus doses would be whether virus infection of capillary endothelial cells in the brain and lysis of these by cytotoxic effector cells occurred.

The requirement for cytotoxic effector function in this process suggests that alternative mechanisms for neuroinvasion (infection of olfactory neurons, transcytosis, or diffusion between capillary endothelial cells) were not involved. However, in mice peripherally infected with a very large virus dose (10^8 PFU), it is likely that the rapid virus entry into the brain occurs by infection of olfactory neurons or diffusion across the blood-brain barrier. The encephalitic flavivirus WNV readily infects human endothelial cells, and the expression of leukocyte adhesion molecules is induced at the plasma membrane of the infected cells (57), which would thus be susceptible to NK/Tc cell-mediated attack. The kinetics of appearance of MVE in the brain (at 6 to 8 days p.i.) following i.v. infection with a low virus dose is consistent with that of the induction of the antiviral Tc cell response (20) and, in turn, Tc cell-induced damage of the cells lining the brain capillaries.

We do not have direct experimental evidence supporting the hypothesis that damage to the integrity of the blood-brain barrier due to the cytotoxic effector pathways allows encephalitic flaviviruses to invade the brain. However, we can exclude the possibility that the brains of *perf*$^{-/-}$ × *gld* mice were resistant to replication of the virus. When a low dose of MVE (10^2 PFU) was injected directly into the brain or when a high dose (10^8 PFU) was injected i.v., all doubly deficient animals, like the wild-type controls, developed fatal encephalitis, and virus titers in the brains of *perf*$^{-/-}$ × *gld* mice were comparable to those in wild-type mice. The induction of virus-specific antibodies in all infected wild-type and mutant mice confirms that infection and extraneural growth of MVE occurred in these animals.

The magnitude of the humoral immune response against MVE determined by ELISA was elevated in mice defective in the Fas receptor (*gld* and *perf*$^{-/-}$ × *gld*) relative to wild-type mice. However, given that the *perf*$^{-/-}$ × *gld* mice were significantly more resistant to MVE infection than *gld* mice, it is unlikely that antibody-mediated virus clearance accounted for the greatly reduced mortality in the former. The ELISA titers of serum samples from infected mice correlated closely with their respective in vitro neutralization activities against MVE measured in plaque reduction neutralization assays (data not shown). Unlike reports on other viral infections (53), the lack of perforin did not alter anti-MVE antibody titers.

The AST of the doubly deficient mice was prolonged by ~2 days relative to that in the wild-type group when infected i.v. with a high virus dose (10^8 PFU). A similar finding was also recorded in a group of *perf* × *gzmAxB*$^{-/-}$ mice and to a lesser extent in groups of *perf*$^{-/-}$ and *gld* mice. This result suggests a second pathogenic consequence of the function of the cytolytic effector pathways of NK/Tc cells in encephalitic flavivirus disease, viz., exacerbation of the contribution of inflammatory infiltration into the CNS of infected mice to a fatal disease outcome.

In summary, this investigation shows the dependence of a neurotropic virus on the major cytotoxic effector pathways for CNS invasion following inoculation of a virus dose by a route analogous to those in natural infections. A deficiency of both the perforin- and Fas-mediated killing pathways was required for this pathogenesis component to become clearly apparent. These findings may pave the way to designing treatments for early times during flavivirus infection to prevent CNS involvement.

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